CELL-SPECIFIC ADENOVIRUS VECTORS COMPRISING AN INTERNAL RIBOSOME ENTRY SITE

TECHNICAL FIELD

This invention relates to new replication competent adenovirus vectors comprising an internal ribosome entry site which replicate preferentially in target cells. The present invention also relates to cell transduction using adenovirus vectors comprising an internal ribosome entry site.

BACKGROUND

Diseases involving altered cell proliferation, particularly hyperproliferation, constitute an important health problem. For example, despite numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Neoplasia resulting in benign tumors can usually be completely cured by surgical removal of the tumor mass. If a tumor becomes malignant, as manifested by invasion of surrounding tissue, it becomes much more difficult to eradicate. Once a malignant tumor metastasizes, it is much less likely to be eradicated.

Excluding basal cell carcinoma, there are over one million new cases of cancer per year in the United States alone, and cancer accounts for over one half million deaths per year in this country. In the world as a whole, the five most common cancers are those of lung, stomach, breast, colon/rectum, and uterine cervix, and the total number of new cases per year is over 6 million.

In the United States, transitional cell carcinoma (TCC) accounts for 90 to 95 percent of all tumors of the bladder. Squamous cell carcinoma (SCC) represents 5 to 10 percent, and adenocarcinoma approximately 1 to 2 percent. Squamous cell and

adenomatous elements are often found in association with transitional cell tumors, especially with high grade tumors. Bladder cancer is generally divided into superficial and invasive disease. A critical factor is the distinction between those tumors that are confined to the mucosa and those that have penetrated the basement membrane and extended into the lamina propria. The term "superficial bladder tumor" is generally used to represent a tumor that has not invaded the muscularis. Invasive tumors are described as those that have invaded the muscularis propria, the perivesical fibroadipose tissue, or adjacent structures. Carcinoma in situ (CIS) is a high grade and aggressive manifestation of TCC of the bladder that has a highly variable course.

A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683). See also, U.S. Patent Nos. 5,824,543 and 6,001,646.

Melanoma, a malignant neoplasm derived from melanocytes of the skin and other sites, has been increasing in incidence worldwide. The American Joint Committee on Cancer recognizes five different forms of extraocular melanoma occurring in humans: lentigo maligna melanoma; radial spreading; nodular; acral

lentiginous; and unclassified. Known melanoma-associated antigens can be classified into three main groups: tumor-associated testis-specific antigens MAGE, BAGE, GAGE, and PRAME; melanocyte differentiation antigens tyrosinase, Melan-A/MART-1 (for Melanoma Antigen Recognized by T cells), gp100, tyrosinase related protein-1(TRP-1), tyrosinase related protein-2 (TRP-2); and mutated or aberrantly expressed antigens MUM-1, cyclin-dependent kinase 4 (CDK4), beta-catenin, gp100-in4, p15, and N-acetylglucosaminyltransferase V. See, for example, Kirkin et al. (1998) *Exp. Clin. Immunogenet.* 15:19-32. Tyrosinase, TRP-1, and TRP-2 are enzymes involved in melanin biosynthesis and are specifically expressed in melanocytes. Antigenic epitopes of MART-1 have been studied extensively, with the aim of developing a melanoma vaccine. An immunodominant epitope, MART-1(27-35) has been reported to be recognized by a majority of CD8+ cytotoxic T cell clones generated to MART-1. These MART-1(27-35)-specific CTLs specifically lyse autologous tumor cell lines expressing the epitope. Faure and Kourilsky (1998) *Crit. Rev. Immunol.* 18:77-86. However, others have reported that presence of such CTLs is not accompanied by a significant clinical response. Rivoltini et al. (1998) *Crit. Rev. Immunol.* 18:55-63.

A major, indeed the overwhelming, obstacle to cancer therapy is the problem of selectivity; that is, the ability to inhibit the multiplication of tumor cells without affecting the functions of normal cells. For example, in traditional chemotherapy of prostate cancer, the therapeutic ratio, (*i.e.*, the ratio of tumor cell killing to normal cell killing) is only 1.5:1. Thus, more effective treatment methods and pharmaceutical compositions for therapy and prophylaxis of neoplasia are needed.

Accordingly, the development of more specific, targeted forms of cancer therapy, especially for cancers that are difficult to treat successfully, is of particular interest. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific treatment modalities, which inhibit or kill malignant cells selectively while leaving healthy cells intact, are required.

Gene therapy, whereby a gene of interest is introduced into a malignant cell, has been attempted as an approach to treatment of many cancers. See, for example, Boulikas (1997) *Anticancer Res.* 17:1471-1505, for a description of gene therapy for prostate cancer. A gene of interest can encode a protein which is converted into a toxic substance upon treatment with another compound, or it can encode an enzyme that converts a prodrug to a drug. For example, introduction of the herpes simplex virus gene encoding thymidine kinase (HSV-tk) renders cells conditionally sensitive to ganciclovir. Zjilstra *et al.* (1989) *Nature* 342: 435; Mansour *et al.* (1988) *Nature* 336: 348; Johnson *et al.* (1989) *Science* 245: 1234; Adair *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 4574; Capecchi (1989) *Science* 244: 1288. Alternatively, a gene of interest can encode a compound that is directly toxic, such as, for example, diphtheria toxin. To render these treatments specific to cancer cells, the gene of interest is placed under control of a transcriptional regulatory element (TRE) that is specifically (*i.e.*, preferentially) active in the cancer cells. Cell- or tissue-specific expression can be achieved by using a TRE with cell-specific enhancers and/or promoters. See generally Huber *et al.* (1995) *Adv. Drug Delivery Reviews* 17:279-292.

A number of viral vectors and non-viral delivery systems (*e.g.*, liposomes), have been developed for gene transfer. Of the viruses proposed for gene transfer, adenoviruses are among the most easily produced and purified. Adenovirus also has the advantage of a high efficiency of transduction (*i.e.*, introduction of the gene of interest into the target cell) and does not require cell proliferation for efficient transduction. In addition, adenovirus can infect a wide variety of cells *in vitro* and *in vivo*. For general background references regarding adenovirus and development of adenoviral vector systems, see Graham *et al.* (1973) *Virology* 52:456-467; Takiff *et al.* (1981) *Lancet* 11:832-834; Berkner *et al.* (1983) *Nucleic Acid Research* 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett *et al.* (1993) *J. Virology* 67:5911-5921; and Bett *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Adenoviruses generally undergo a lytic replication cycle following infection of a host cell. In addition to lysing the infected cell, the replicative process of adenovirus blocks the transport and translation host cell mRNA, thus inhibiting cellular protein synthesis. For a review of adenoviruses and adenovirus replication, see Shenk, T. and Horwitz, M.S., *Virology*, third edition, Fields, B.N. *et al.*, eds., Raven Press Limited, New York (1996), Chapters 67 and 68, respectively.

When used for gene transfer, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cell. In these vectors, the early adenovirus gene products E1A and/or E1B are often deleted, and the gene to be transduced is commonly inserted into the E1A and/or E1B region of the deleted virus genome. Bett *et al.* (1994) *supra*. Such vectors are propagated in packaging cell lines such as the 293 line, which provides E1A and E1B functions in *trans*. Graham *et al.* (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Gen. Virol.* 68:937-940. The use of replication-defective adenovirus vectors as vehicles for efficient transduction of genes has been described by, *inter alia*, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang *et al.* (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe *et al.* (1992) *Nature Gen.* 1:372-378; Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld *et al.* (1992) *Cell* 68:143-155; Stratford-Perricaudet *et al.* (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle *et al.* (1993) *Science* 259:988-990; Mastrangeli *et al.* (1993) *J. Clin. Invest.* 91:225-234; Ragot *et al.* (1993) *Nature* 361:647-650; Hayaski *et al.* (1994) *J. Biol. Chem.* 269:23872-23875; and Bett *et al.* (1994) *supra*.

In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes,

suicide genes, or genes which convert a prodrug to an active drug has been limited by the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle, coupled with the transient nature of gene expression. Despite these limitations, development of adenoviral vectors for gene therapy has focused almost exclusively on the use of the virus as a vehicle for introducing a gene of interest, not as an effector in itself. In fact, replication of adenovirus vectors has been viewed as an undesirable result, largely due to the host immune response.

More recently, however, the use of adenovirus vectors as effectors has been described. International Patent Application Nos. PCT/US98/04080, PCT/US98/04084, PCT/US98/04133, PCT/US98/04132, PCT/US98/16312, PCT/US95/00845, PCT/US96/10838, PCT/EP98/07380 and U.S. Pat. No. 5,998,205. Adenovirus E1A and E1B genes are disclosed in Rao et al. (1992, *Proc. Natl. Acad. Sci.* USA vol. 89: 7742-7746).

Replication-competent adenovirus vectors, which take advantage of the cytotoxic effects associated with adenovirus replication, have recently been described as agents for effecting selective cell growth inhibition. In such systems, a cell-specific transcriptional regulatory element (TRE) is used to control the expression of a gene essential for viral replication, thus limiting viral replication to cells in which the TRE is functional. *See*, for example International Patent Application No. PCT/EP99/07380, Henderson *et al.*, U.S. Patent No. 5,698,443; Hallenbeck *et al.*, PCT/US95/15455 and U.S. Patent No. 5,998,205; Rodriguez *et al.* (1997) *Cancer Res.* 57:2559-2563.

PCT publication PCT/US98/04080 discloses replication-competent, target cell-specific adenovirus vectors comprising heterologous TREs, such as those regulating expression of prostate-specific antigen (PSA), probasin (PB), α-fetoprotein (AFP), kallikrien (*hKLK2*), mucin (*MUC1*) and carcinoembryonic antigen (CEA). PCT/US98/04084 discloses replication-competent adenovirus vectors comprising an

α-fetoprotein (AFP) TRE that replicate specifically in cells expressing AFP, such as hepatoma cells.

Internal ribosome entry sites (IRES) are sequences which initiate translation from an internal initiation codon (usually AUG) within a bi-or multi- cistronic RNA transcript continuing multiple protein coding regions. IRES have been characterized in encephalomyocarditis virus and related picornaviruses. *See*, for example, Jackson *et al.* (1995) *RNA* **1:** 985-1000 and Herman (1989) *Trends in Biochemical Sciences* 14(6): 219-222. IRES sequences are also detected in mRNAs from other viruses such as cardiovirus, rhinovirus, aphthovirus, hepatitis C virus (HCV), Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). The presence of IRES in cellular RNAs has also been described. Examples of cellular mRNAs containing IRES include those encoding immunoglobulin heavy-chain binding protein (BiP), vascular endothelial growth factor (VEGF), fibroblast growth factor 2, insulin-like growth factor, translational initiation factor eIF4G, and the yeast transcription factors TFIID and HAP4. *See*, for example, ; Macejak *et al.* (1991) *Nature* **353:**90-94; Oh *et al.* (1992) *Genes Dev.* **6:**1643-1653; Vagner *et al.* (1995) *Mol. Cell. Biol.* **15:**35-44; He *et al.* (1996) *Proc. Natl. Acad. Sci USA* **93:**7274-7278; He *et al.* (1996) *Gene* **175:**121-125; Tomanin *et al.* (1997) *Gene* **193:**129-140; Gambotto *et al.* (1999) *Cancer Gene Therapy* **6:**45-53; Qiao *et al.* (1999) *Cancer Gene Therapy* **6:**373-379. Expression vectors containing IRES elements have been described. *See*, for example, International Patent Application No. PCT/US98/03699 and International Patent Application No. PCT/EP98/07380.

Thus, there is a continuing need for improved replication-competent adenovirus vectors in which cell-specific replication can be further enhanced, while minimizing the extent of replication in non-target (*i.e.*, non-cancerous cells).

The disclosure of all patents and publications cited herein are incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention provides improved replication competent adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site (IRES). In one embodiment, the first and second genes are co-transcribed as a single mRNA and the second gene has a mutation in or deletion of its endogenous promoter. The present invention further provides host cells and methods using the adenovirus vectors.

In one aspect, the first and/or second genes are adenovirus genes and in another aspect, the first and/or second adenovirus genes are essential for viral replication. An essential gene can be an early viral gene, including for example, E1A; E1B; E2; and/or E4, or a late viral gene. In another aspect an early gene is E3.

In one embodiment, the first gene is an adenovirus gene and the second gene is a therapeutic gene. In another embodiment, both genes are adenovirus genes. In an additional embodiment, the first adenovirus gene is E1A, and the second adenovirus gene is E1B. Optionally, the endogenous promoter for one of the co-transcribed adenovirus gene essential for viral replication, such as for example, E1A, is deleted and/or mutated such that the gene is under sole transcriptional control of a target cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising an adenovirus gene essential for viral replication under control of a target cell-specific TRE, wherein said adenovirus gene has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus gene is essential for viral replication. In another embodiment, the adenovirus gene is E1A wherein the E1A promoter is deleted and wherein the E1A gene is under transcriptional control of a heterologous cell-specific TRE. In another embodiment, the adenovirus gene is E1B

wherein the E1B promoter is deleted and wherein the E1B gene is under transcriptional control of a heterologous cell-specific TRE.

In another aspect, the present invention provides adenovirus vectors comprising E1B under control of a target cell-specific TRE, wherein said E1B has a deletion in or mutation of the 19-kDa region of E1B, that encodes a product shown to inhibit apoptosis.

In other embodiments, an enhancer element for the first and/or second adenovirus genes is inactivated. The present invention provides an adenovirus vector comprising E1A wherein an E1A enhancer is inactivated. In yet other embodiments, the present invention provides an adenovirus vector comprising E1A wherein the E1A promoter is inactivated and E1A enhancer I is inactivated. In further embodiments, the present invention provides an adenovirus vector comprising a TRE which has its endogenous silencer element inactivated.

Any TRE which directs cell-specific expression can be used in the disclosed vectors. In one embodiment, TREs include, for example, TREs specific for prostate cancer cells, breast cancer cells, hepatoma cells, melanoma cells, bladder cells and/or colon cancer cells. In another embodiment, the TREs include, probasin (PB) TRE; prostate-specific antigen (PSA) TRE; mucin (*MUC1*) TRE; α-fetoprotein (AFP) TRE; *hKLK2* TRE; tyrosinase TRE; human uroplakin II TRE (hUPII) and carcinoembryonic antigen (CEA) TRE. In other embodiments, the target cell-specific TRE is a cell status-specific TRE. In yet other embodiments, the target cell-specific TRE is a tissue specific TRE.

In additional embodiments, the adenovirus vector comprises at least one additional co-transcribed gene under the control of the cell-specific TRE. In another embodiment, an additional co-transcribed gene is under the translational control of an IRES.

In another aspect of the present invention, adenovirus vectors further comprise a transgene such as, for example, a cytotoxic gene. In one embodiment, the

transgene is under the transcriptional control of the same TRE as the first gene and second genes and optionally under the translational control of an internal ribosome entry site. In another embodiment, the transgene is under the transcriptional control of a different TRE that is functional in the same cell as the TRE regulating transcription of the first and second genes and optionally under the translational control of an IRES.

The present invention also provides compositions comprising the replication-competent adenovirus vectors described herein. In one embodiment, the compositions further comprise a pharmaceutically acceptable excipient. The present invention also provides kits comprising the replication-competent adnenovirus vectors described herein.

Host cells comprising the disclosed adenovirus vectors are also provided. Host cells include those used for propagation of a vector and those into which a vector is introduced for therapeutic purposes.

In another aspect, methods are provided for propagating replication-competent adenovirus vectors of the present invention specific for mammalian cells which permit the function of a target cell-specific TRE, said method comprising combining an adenovirus vector(s) described herein with mammalian cells that permit the function of a target cell-specific TRE, such that the adenovirus vector(s) enters the cell, whereby said adenovirus is propagated.

In another aspect, methods are provided for conferring selective cytotoxicity in target cells, comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, more particularly a target tumor cell, comprising contacting a tumor cell with an adenovirus vector(s) of the invention such that the adenovirus vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell.

In another aspect, methods are provided for detecting a cell which allows the function of a target cell-specific TRE, which comprise contacting a cell in a biological sample with an adenovirus vector(s) of the invention, and detecting replication of the adenovirus vector(s), if any.

In another aspect, methods are provided for modifying the genotype of a target cell, comprising contacting the cell with an adenovirus vector as described herein, wherein the adenovirus vector enters the cell.

The present invention provides an adenovirus vector comprising an adenovirus gene, wherein said adenovirus gene is under transcriptional control of a melanocyte-specific TRE. In another embodiment, a melanocyte-specific TRE is human. In another embodiment, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a heterologous enhancer. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific enhancer and a heterologous promoter. In other embodiments, a melanocyte-specific TRE comprises a melanocyte-specific promoter and a melanocyte-specific enhancer.

In some embodiments, the adenovirus gene under transcriptional control of a melanocyte-specific TRE is an adenovirus gene essential for replication. In some embodiments, the adenoviral gene essential for replication is an early gene. In another embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a melanocyte-specific TRE. In further embodiments, the adenovirus gene essential for replication is E1B, and E1B has a deletion in the 19-kDa region.

In some embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a tyrosinase gene. In other embodiments, the melanocyte-specific TRE is derived from the 5' flanking region of a tyrosinase related protein-1 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5'-flanking region of a tyrosinase related protein-2 gene. In other embodiments, the melanocyte-

specific TRE is derived from the 5' flanking region of a MART-1 gene. In other embodiments, the melanocyte-specific TRE is derived from the 5'-flanking region of a gene which is aberrantly expressed in melanomas.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a melanocyte-specific TRE; and (b) an E3 region. In some of these embodiments the E3 region is under transcriptional control of a melanocyte-specific TRE.

In another aspect, the invention provides a host cell comprising the melanocyte specific adenovirus vector(s) described herein.

In another aspect, the invention provides pharmaceutical compositions comprising a melanocyte specific adenovirus vector(s) described herein.

In another aspect, the invention provides kits which contain a melanocyte adenoviral vector(s) described herein.

In another aspect, methods are provided for conferring selective cytoxicity in target cells (i.e., cells which permit or induce a melanocyte-specific TRE to function), comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

In another aspect, methods are provided for propagating an adenovirus specific for melanocytes, said method comprising combining an melanocyte specific adenovirus vector(s) described herein with melanocytes, whereby said adenovirus is propagated.

The invention further provides methods of suppressing melanoma cell growth, comprising contacting a melanoma cell with a melanocyte specific adenoviral vector of the invention such that the adenoviral vector enters the melanoma cell and exhibits selective cytotoxicity for the melanoma cell.

In another aspect, methods are provided for detecting melanocytes, including melanoma cells, in a biological sample, comprising contacting cells of a biological

sample with a melanocyte adenovirus vector(s) described herein, and detecting replication of the adenovirus vector, if any.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of plasmid construct CP627 as described in Example 1.

Figures 2A-2B is a series of schematic depictions of various adenoviruses described herein.

Figure 3 depicts the replication efficiency of different viruses as described in Example 4.

Figures 4A and 4B show viral yield for different liver-specific vectors in different cell types.

Figure 5 is a schematic representation of adenovirus vectors comprising AFP-TRE with and without IRES.

Figure 6 depicts an E3 region.

Figure 7 is a schematic representation of adenovirus vectors described herein.

Figure 8 depicts in vivo antitumor activity of CV890 containing an IRES. This figure depicts the results of a HepG2 Xenograph treated with CV790 or CV890.

Figure 9 depicts an ADP nucleotide and amino acid sequence.

Figure 10 depicts an IC_{50} isobologram of doxorubicin and CV 890 on Hep3B cells at day 5.

Figure 11 depicts in vivo efficacy of CV890 with doxorubicin. Hep3B nude mouse xenografts were grouped (n=6) and treated with CV890 alone ($1x10^{11}$ particles/dose, iv), doxorubicin alone (10mg/kg, ip), CV890 and doxorubicin combination ($1x10^{11}$ particles of CV890 through tail vein and 10mg/kg doxorubicin ip), or vehicle control. Tumor size was measured weekly and the tumor volume were normalized as 100% at the day of treatment. Error bars represent the standard error of the mean.

Figure 12 shows the virus yield of CV802, CV882 and CV884 in cell lines.

MODES FOR CARRYING OUT THE INVENTION

We have discovered and constructed improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific transcriptional regulatory element (TRE), wherein the second gene is under translational control of an internal ribosome entry site (IRES). In one embodiment, the first and second genes are co-transcribed as a single mRNA and the second gene has a mutation in or deletion of its endogenous promoter. In another embodiment, at least one of the genes is an adenovirus gene and in yet another embodiment, both genes are adenovirus genes, including adenovirus genes that are essential for viral replication. The adenovirus vector may comprise a gene that contributes to cytotoxicity (whether direct and/or indirect), and/or causes cell death. An example of an adenovirus gene that contributes to cytotoxicity includes, but is not limited to, the adenovirus death protein gene.

In some aspects of the present invention, an adenovirus vector comprising co-transcribed first and second genes under transcriptional control of a target cell-specific TRE, wherein the second gene is under translational control of an IRES, exhibits greater specificity for the target cell than an adenovirus vector comprising a target cell-specific TRE operably linked to a gene and lacking an IRES. In some embodiments, specificity is conferred by preferential transcription and/or translation of the first and second genes due to the presence of a target cell specific TRE. In other embodiments, specificity is conferred by preferential replication of the adenovirus vectors in target cells due to the target cell-specific TRE driving transcription of a gene essential for replication.

Also disclosed herein are IRES containing adenovirus vectors comprising an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion of its endogenous promoter. In an embodiment disclosed

herein, the adenovirus vectors comprise the adenovirus early gene E1A which has a deletion of its endogenous promoter. In another embodiment disclosed herein, the adenovirus vectors comprise the adenovirus early gene E1B which has a deletion of its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

In another aspect, the adenovirus vectors disclosed herein comprise an adenovirus gene essential for viral replication wherein said essential gene has a mutation in or deletion of its endogenous enhancer. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter. In one embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in E1A enhancer 1. In a further embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter and a mutation of or deletion in the E1A enhancer. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A which has a mutation of or deletion in its endogenous promoter and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In an additional embodiment, the adenovirus vector comprises the adenovirus early gene E1A, which has a mutation of or deletion in its endogenous promoter and a mutation of or deletion in the E1A enhancer I, and the adenovirus early gene E1B which has a mutation of or deletion in its endogenous promoter. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

The replication-competent adenovirus vectors of the present invention take advantage of what has been heretofore considered an undesirable aspect of adenovirus vectors, namely their replication and possible concomitant immunogenicity. Runaway infection is prevented due to the cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, it is noted that production of adenovirus proteins can serve to activate and/or

stimulate the immune system, either generally or specifically, toward target cells producing adenoviral proteins. This type of immune stimulation can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

The adenovirus vectors of the present invention comprising an intergenic IRES element(s) which links the translation of two or more genes, reflects an improvement over vector constructs which use identical control regions to drive expression of two or more desired genes in that any potential for homologous recombination based on the presence of homologous control regions in the vector is removed. As demonstrated herein, adenovirus vectors comprising an IRES are stable and in some embodiments provide better specificity than vectors not containing an IRES. Another advantage of an adenovirus vector comprising an intergenic IRES is that the use of an IRES rather than a second TRE may provide additional space in the vector for an additional gene(s) such as a therapeutic gene.

Thus, the adenovirus vectors comprising a second gene under control of an IRES retain a high level of target cell specificity and remain stable in the target cell. Accordingly, in one aspect of the invention, the viral vectors disclosed herein comprise at least one IRES within a multicistronic transcript, wherein production of the multicistronic transcript is regulated by a heterologous, target cell-specific TRE. For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and the second gene are in frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used. In one embodiment, the adenovirus

vectors comprises the adenovirus essential genes, E1A and E1B genes, under the transcriptional control of a heterologous, cell-specific TRE, and an IRES introduced between E1A and E1B. Thus, both E1A and E1B are under common transcriptional control, and translation of E1B coding region is obtained by virtue of the presence of the IRES. In one embodiment, E1A has its endogenous promoter deleted. In another embodiment, E1A has an endogenous enhancer deleted and in yet an additional embodiment, E1A has its endogenous promoter deleted and E1A enhancer I deleted. In another embodiment, E1B has its endogenous promoter deleted. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted.

To provide cytotoxicity to target cells, one or more transgenes having a cytotoxic effect may be present in the vector. Additionally, or alternatively, an adenovirus gene that contributes to cytotoxicity and/or cell death, such as the adenovirus death protein (ADP) gene, can be included in the vector, optionally under the selective transcriptional control of a heterologous TRE and optionally under the translational control of an IRES.

Examples of target cells include neoplastic cells, although any cell for which it is desirable and/or tolerable to sustain a cytotoxic activity can be a target cell. By combining an adenovirus vector(s) comprising a target cell-specific TRE with a mixture of target and non-target cells, *in vitro* or *in vivo,* the vector(s) preferentially replicates in the target cells, causing cytotoxic and/or cytolytic effects. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic activity, replication of the vector(s) is significantly reduced, lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures can be retained to continually monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for the presence of the undesirable target cell, *e.g.*, a cancer cell in which the target cell-specific TRE is functional. The adenovirus vectors of the present invention can also be used in *ex vivo* procedures wherein desirable biological samples comprising target cells are removed from the animal,

subjected to exposure to an adenovirus vector of the present invention comprising a target cell-specific TRE and then replaced within the animal.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook *et al.*, 1989); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Animal Cell Culture* (R.I. Freshney, ed., 1987); *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D.M. Wei & C.C. Blackwell, eds.); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller & M.P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F.M. Ausubel *et al.*, eds., 1987 and annual updates); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991 and annual updates).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

As used herein, an "internal ribosome entry site" or "IRES" refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention encompasses the use of any IRES element which is able to promote direct internal ribosome entry to the initiation codon of a cistron. "Under translational control of an IRES" as used herein means that translation is associated

with the IRES and proceeds in a cap-independent manner. Examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson et al., 1990, *Trends Biochem Sci* 15(12):477-483); and IRES obtainable from viral or cellular mRNA sources, such as for example, immunogloublin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez et al. (1998) *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2, and insulin-like growth factor, the translational initiation factor eIF4G, yeast transcription factors TFIID and HAP4. IRES have also been reported in different viruses such as cardiovirus, rhinovirus, aphthovirus, HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). As used herein, "IRES" encompasses functional variations of IRES sequences as long as the variation is able to promote direct internal ribosome entry to the initiation codon of a cistron. In preferred embodiments, the IRES is mammalian. In other embodiments, the IRES is viral or protozoan. In one illustrative embodiment disclosed herein, the IRES is obtainable from encephelomycarditis virus (ECMV) (commercially available from Novogen, Duke et al. (1992) *J. Virol* 66(3):1602-1609). In another illustrative embodiment disclosed herein, the IRES is from VEGF. Table I and Table II disclose a variety of IRES sequences useful in the present invention.

A "multicistronic transcript" refers to an mRNA molecule which contains more than one protein coding region, or cistron. A mRNA comprising two coding regions is denoted a "bicistronic transcript." The "5'-proximal" coding region or cistron is the coding region whose translation initiation codon (usually AUG) is closest to the 5'-end of a multicistronic mRNA molecule. A "5'-distal" coding region or cistron is one whose translation initiation codon (usually AUG) is not the closest initiation codon to the 5' end of the mRNA. The terms "5'-distal" and "downstream" are used synonymously to refer to coding regions that are not adjacent to the 5' end of a mRNA molecule.

As used herein, "co-transcribed" means that two (or more) coding regions of polynucleotides are under transcriptional control of single transcriptional control element.

A "gene" refers to a coding region of a polynucleotide. A "gene" may or may not include non-coding sequences and/or regulatory elements.

As used herein, a "transcription response element" or "transcriptional regulatory element", or "TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows that TRE to function. A TRE can comprise an enhancer and/or a promoter. A "transcriptional regulatory sequence" is a TRE. A "target cell-specific transcriptional response element" or "target cell-specific TRE" is a polynucleotide sequence, preferably a DNA sequence, which is preferentially functional in a specific type of cell, that is, a target cell. Accordingly, a target cell-specific TRE transcribes an operably linked polynucleotide sequence in a target cell that allows the target cell-specific TRE to function. The term "target cell-specific", as used herein, is intended to include cell type specificity, tissue specificity, developmental stage specificity, and tumor specificity, as well as specificity for a cancerous state of a given target cell. "Target cell-specific TRE" includes cell type-specific and cell status-specific TRE, as well as "composite" TREs. The term "composite TRE" includes a TRE which comprises both a cell type-specific and a cell status-specific TRE. A target cell-specific TRE can also include a heterologous component, including, for example, an SV40 or a cytomegalovirus (CMV) promoter(s). An example of a target cell specific TRE which is tissue specific is a CMV TRE which contains both promoter(s) and enhancer(s).

As described in more detail herein, a target cell-specific TRE can comprise any number of configurations, including, but not limited to, a target cell-specific promoter; and target cell-specific enhancer; a heterologous promoter and a target cell-specific enhancer; a target cell-specific promoter and a heterologous enhancer; a

heterologous promoter and a heterologous enhancer; and multimers of the foregoing. The promoter and enhancer components of a target cell-specific TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired target cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the target cell-specific TRE. As discussed herein, a target cell-specific TRE can be of varying lengths, and of varying sequence composition. As used herein, the term "cell status-specific TRE" is preferentially functional, i.e., confers transcriptional activation on an operably linked polynucleotide in a cell which allows a cell status-specific TRE to function, i.e., a cell which exhibits a particular physiological condition, including, but not limited to, an aberrant physiological state. "Cell status" thus refers to a given, or particular, physiological state (or condition) of a cell, which is reversible and/or progressive. The physiological state may be generated internally or externally; for example, it may be a metabolic state (such as in response to conditions of low oxygen), or it may be generated due to heat or ionizing radiation. "Cell status" is distinct from a "cell type", which relates to a differentiation state of a cell, which under normal conditions is irreversible. Generally (but not necessarily), as discussed herein, a cell status is embodied in an aberrant physiological state, examples of which are given below.

A "functional portion" of a target cell-specific TRE is one which confers target cell-specific transcription on an operably linked gene or coding region, such that the operably linked gene or coding region is preferentially expressed in the target cells.

By "transcriptional activation" or an "increase in transcription," it is intended that transcription is increased above basal levels in the target cell (i.e., target cell) by at least about 2 fold, preferably at least about 5 fold, preferably at least about 10 fold,

more preferably at least about 20 fold, more preferably at least about 50 fold, more preferably at least about 100 fold, more preferably at least about 200 fold, even more preferably at least about 400 fold to about 500 fold, even more preferably at least about 1000 fold. Basal levels are generally the level of activity (if any) in a non-target cell (i.e., a different cell type), or the level of activity (if any) of a reporter construct lacking a target cell-specific TRE as tested in a target cell line.

A "functionally-preserved variant" of a target cell-specific TRE is a target cell-specific TRE which differs from another target cell-specific TRE, but still retains target cell-specific transcription activity, although the degree of activation may be altered (as discussed below). The difference in a target cell-specific TRE can be due to differences in linear sequence, arising from, for example, single base mutation(s), addition(s), deletion(s), and/or modification(s) of the bases. The difference can also arise from changes in the sugar(s), and/or linkage(s) between the bases of a target cell-specific TRE. For example, certain point mutations within sequences of TREs have been shown to decrease transcription factor binding and stimulation of transcription. See Blackwood, et al. (1998) *Science* 281:60-63 and Smith et al. (1997) *J. Biol. Chem.* 272:27493-27496. One of skill in the art would recognize that some alterations of bases in and around transcription factor binding sites are more likely to negatively affect stimulation of transcription and cell-specificity, while alterations in bases which are not involved in transcription factor binding are not as likely to have such effects. Certain mutations are also capable of increasing TRE activity. Testing of the effects of altering bases may be performed *in vitro* or *in vivo* by any method known in the art, such as mobility shift assays, or transfecting vectors containing these alterations in TRE functional and TRE non-functional cells. Additionally, one of skill in the art would recognize that point mutations and deletions can be made to a TRE sequence without altering the ability of the sequence to regulate transcription.

As used herein, a TRE derived from a specific gene is referred to by the gene from which it was derived and is a polynucleotide sequence which regulates transcription of an operably linked polynucleotide sequence in a host cell that expresses said gene. For example, as used herein, a "human glandular kallikrein transcriptional regulatory element", or "*hKLK2*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows an *hKLK2*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses androgen receptor, such as a prostate cell. An *hKLK2*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of an *hKLK2* promoter and/or an *hKLK2* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "probasin (*PB*) transcriptional regulatory element", or "*PB*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably-linked polynucleotide sequence in a host cell that allows a *PB*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PB*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a *PB* promoter and/or a *PB* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "prostate-specific antigen (*PSA*) transcriptional regulatory element", or "*PSA*-TRE", or "*PSE*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *PSA*-TRE to function, such as a cell (preferably a mammalian cell, more preferably a human cell, even more preferably a prostate cell) that expresses androgen receptor. A *PSA*-TRE is thus responsive to the binding of androgen receptor and comprises at least a portion of a

PSA promoter and/or a *PSA* enhancer (i.e., the ARE or androgen receptor binding site).

As used herein, a "carcinoembryonic antigen (*CEA*) transcriptional regulatory element", or "*CEA*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription of an operably linked polynucleotide sequence in a host cell that allows a *CEA*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses CEA. The *CEA*-TRE is responsive to transcription factors and/or co-factor(s) associated with CEA-producing cells and comprises at least a portion of the *CEA* promoter and/or enhancer.

As used herein, an "α-fetoprotein (*AFP*) transcriptional regulatory element", or "*AFP*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably linked polynucleotide sequence) in a host cell that allows an *AFP*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses AFP. The *AFP*-TRE is responsive to transcription factors and/or co-factor(s) associated with AFP-producing cells and comprises at least a portion of the *AFP* promoter and/or enhancer.

As used herein, an "a mucin gene (*MUC*) transcriptional regulatory element", or "*MUC1*-TRE" is a polynucleotide sequence, preferably a DNA sequence, which selectively increases transcription (of an operably-linked polynucleotide sequence) in a host cell that allows a *MUC1*-TRE to function, such as a cell (preferably a mammalian cell, even more preferably a human cell) that expresses MUC1. The *MUC1*-TRE is responsive to transcription factors and/or co-factor(s) associated with MUC1-producing cells and comprises at least a portion of the *MUC1* promoter and/or enhancer.

As used herein, a "urothelial cell-specific transcriptional response element", or "urothelial cell-specific TRE" is polynucleotide sequence, preferably a DNA

sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a urothelial-specific TRE to function, i.e., a target cell. A variety of urothelial cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with urothelial cells, and comprise at least a portion of a urothelial-specific promoter and/or a urothelial-specific enhancer. Methods are described herein for measuring the activity of a urothelial cell-specific TRE and thus for determining whether a given cell allows a urothelial cell-specific TRE to function.

As used herein, a "melanocyte cell-specific transcriptional response element", or "melanocyte cell-specific TRE" is polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a melanocyte-specific TRE to function, i.e., a target cell. A variety of melanocyte cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocyte cells, and comprise at least a portion of a melanocyte-specific promoter and/or a melanocyte-specific enhancer. Methods are described herein for measuring the activity of a melanocyte cell-specific TRE and thus for determining whether a given cell allows a melanocyte cell-specific TRE to function.

An "E1B 19-kDa region" (used interchangeably with "E1B 19-kDa genomic region") refers to the genomic region of the adenovirus E1B gene encoding the E1B 19-kDa product. According to wild-type Ad5 , the E1B 19-kDa region is a 261bp region located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA, 89:*7742-7746. The present invention encompasses deletion of part or all of the E1B 19-kDa region as well as embodiments wherein the E1B 19-kDa region is mutated, as long as the deletion or mutation lessens or eliminates the inhibition of apoptosis associated with E1B-19kDa.

As used herein, a target cell-specific TRE can comprise any number of configurations, including, but not limited to, a target cell-specific promoter; a target cell-specific enhancer; a target cell-specific promoter and a target cell-specific enhancer; a target cell-specific promoter and a heterologous enhancer; a heterologous promoter and a target cell-specific enhancer; and multimers of the foregoing. The promoter and enhancer components of a target cell-specific TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired target cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the target cell-specific TRE.

"Replicating preferentially", as used herein, means that the adenovirus replicates more in a target cell than a non-target cell. Preferably, the adenovirus replicates at a significantly higher rate in target cells than non target cells; preferably, at least about 2-fold higher, preferably, at least about 5-fold higher, more preferably, at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400- to 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1 x 10^6 higher. Most preferably, the adenovirus replicates solely in the target cells (that is, does not replicate or replicates at a very low levels in non-target cells).

As used herein, the term "vector" refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, or a "viral vector" which is

designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector.

An "adenovirus vector" or "adenoviral vector" (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, and conjugated to a nonviral protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate- phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-

23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothioate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Molec. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. Reference to a polynucleotide sequence (such as referring to a SEQ ID NO) also includes the complement sequence.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means

that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2.

"Under transcriptional control" is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. "Operably linked" refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

An "E3 region" (used interchangeably with "E3") is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 products (discussed herein). Generally, the E3 region is located between about 28583 and 30470 of the adenoviral genome. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

A "portion" of the E3 region means less than the entire E3 region, and as such includes polynucleotide deletions as well as polynucleotides encoding one or more polypeptide products of the E3 region.

As used herein, "cytotoxicity" is a term well understood in the art and refers to a state in which a cell's usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular replication; DNA replication; transcription; translation; uptake of molecules. "Cytotoxicity" includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ^{3}H-thymidine uptake, and plaque assays.

The term "selective cytotoxicity", as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or induces a target cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a target cell-specific TRE to function (a non-target cell). Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker.

In the context of adenovirus, a "heterologous polynucleotide" or "heterologous gene" or "transgene" is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

In the context of adenovirus, a "heterologous" promoter or enhancer is one which is not associated with or derived from an adenovirus gene.

In the context of adenovirus, an "endogenous" promoter, enhancer, or TRE is native to or derived from adenovirus. In the context of promoter, an "inactivation" means that there is a mutation of or deletion in part or all of the of the endogenous promoter, ie, a modification or alteration of the endogenous promoter, such as, for example, a point mutation or insertion, which disables the function of the promoter.

In the context of a target cell-specific TRE, a "heterologous" promoter or enhancer is one which is derived from a gene other than the gene from which a reference target cell-specific TRE is derived.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells

are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A "host cell" includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

"Replication" and "propagation" are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. "Replication" and "propagation" include any activity directly or indirectly involved in the process of virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

An "ADP coding sequence" is a polynucleotide that encodes ADP or a functional fragment thereof. In the context of ADP, a "functional fragment" of ADP is one that exhibits cytotoxic activity, especially cell lysis, with respect to adenoviral

replication. Ways to measure cytotoxic activity are known in the art and are described herein.

A polynucleotide that "encodes" an ADP polypeptide is one that can be transcribed and/or translated to produce an ADP polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

An "ADP polypeptide" is a polypeptide containing at least a portion, or region, of the amino acid sequence of an ADP and which displays a function associated with ADP, particularly cytotoxicity, more particularly, cell lysis. As discussed herein, these functions can be measured using techniques known in the art. It is understood that certain sequence variations may be used, due to, for example, conservative amino acid substitutions, which may provide ADP polypeptides.

"Androgen receptor," or AR, as used herein refers to a protein whose function is to specifically bind to androgen and, as a consequence of the specific binding, recognize and bind to an androgen response element (ARE), following which the AR is capable of regulating transcriptional activity. The AR is a nuclear receptor that, when activated, binds to cellular androgen-responsive element(s). In normal cells the AR is activated by androgen, but in non-normal cells (including malignant cells) the AR may be activated by non-androgenic agents, including hormones other than androgens. Encompassed in the term "androgen receptor" are mutant forms of an androgen receptor, such as those characterized by amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. Mutants include androgen receptors with amino acid additions, insertions, truncations and deletions, as long as the function is sufficiently preserved. In this context, a functional androgen receptor is one that binds both androgen and, upon androgen binding, an ARE.

A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the sequence is present as an identical contiguous sequence in the SEQ ID NO. The term

encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets.

An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

A given TRE is "derived from" a given gene if it is associated with that gene in nature.

"Expression" includes transcription and/or translation.

As used herein, the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

"A," "an" and "the" include plural references unless the context clearly dictates otherwise.

Internal Ribosome Entry Site (IRES)

IRES elements were first discovered in picornavirus mRNAs (Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention provides improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific TRE, and wherein the second gene (i.e., coding region) is under translational control of an internal ribosome entry site (IRES). Any IRES may be used in the adenovirus vectors of the invention, as long as they exhibit requisite function in the vectors. Example of IRES which can be used in the present invention include those provided in Table I and referenced in Table II. Examples of IRES elements include the encephelomycarditis virus (EMCV) which is commercially available from Novagen (Duke et al. (1992) *J. Virol* 66(3):1602-9) the sequence for which is depicted in Table 1 (SEQ ID NO:1). Another example of an IRES element disclosed herein is the VEGF IRES (Huez et al. (1998) *Mol Cell Biol* 18(11):6178-90). This IRES has a short segment and the sequence is depicted in Table 1 (SEQ ID NO:2).

The IRES promotes direct internal ribosome entry to the initiation codon of a downstream cistron, leading to cap-independent translation. Thus, the product of a downstream cistron can be expressed from a bicistronic (or multicistronic) mRNA, without requiring either cleavage of a polyprotein or generation of a monocistronic mRNA. Therefore, in one illustrative embodiment of the present invention, an adenovirus vector comprising E1B under translational control of an IRES allows translation of E1B from a bicistronic E1A-E1B mRNA under control of a target cell-specific TRE. Figure 7 provides a schematic representation of adenovirus constructs of the present invention.

Internal ribosome entry sites are approximately 450 nucleotides in length and are characterized by moderate conservation of primary sequence and strong conservation of secondary structure. The most significant primary sequence feature

of the IRES is a pyrimidine-rich site whose start is located approximately 25 nucleotides upstream of the 3' end of the IRES. *See* Jackson *et al.(1990).*

Three major classes of picornavirus IRES have been identified and characterized: (1) the cardio- and aphthovirus class (for example, the encephelomycarditis virus, Jang et al. (1990) *Gene Dev* 4:1560-1572); (2) the entero- and rhinovirus class (for example, polioviruses, Borman et al. (1994) *EMBO J.* 13:314903157); and (3)the hepatitis A virus (HAV) class, Glass et al. (1993) *Virol* 193:842-852). For the first two classes, two general principles apply. First, most of the 450-nucleotide sequence of the IRES functions to maintain particular secondary and tertiary structures conducive to ribosome binding and translational initiation. Second, the ribosome entry site is an AUG triplet located at the 3' end of the IRES, approximately 25 nucleotides downstream of a conserved oligopyrimidine tract. Translation initiation can occur either at the ribosome entry site (cardioviruses) or at the next downstream AUG (entero/rhinovirus class). Initiation occurs at both sites in aphthoviruses.

HCV and pestiviruses such as bovine viral diarrhea virus (BVDV) or classical swine fever virus (CSFV) have 341 nt and 370 nt long 5'-UTR respectively. These 5'-UTR fragments form similar RNA secondary structures and can have moderately efficient IRES function (Tsukiyama-Kohara et al. (1992) *J. Virol.* 66:1476-1483; Frolov I et al., (1998) *RNA* 4:1418-1435). Table I depicts the 5'-UTR region from HCV genome sequence (GenBank accession D14853).

Leishmania RNA virus 1 (LRV1) is a double-stranded RNA virus. Its 128 nt long 5'-UTR has IRES activity to facilitate the cap-independent translation, (Maga et al. (1995) *Mol Cell Biol* 15:4884-4889). This fragment also forms conserved stemloop secondary structure and at least the front part is essential.

Recent studies showed that both Friend-murine leukemia virus (MLV) 5'-UTR and rat retrotransposon virus-like 30S (VL30) sequences contain IRES structure of retroviral origin (Torrent et al. (1996) *Hum Gene Ther* 7:603-612). These

fragments are also functional as packing signal when used in retrovirus derived vectors. Studies of avian reticuloendotheliosis virus type A (REV-A) show that its IRES maps downstream of the packaging/dimerization (E/DLS) sequence and the minimal IRES sequence appears to be within a 129 nt fragment (452-580) of the 5' leader, immediately upstream of the gag AUG codon (Lopez-Lastra et al. (1997) *Hum Gene Ther* 8:1855-1865).

In eukaryotic cells, translation is normally initiated by the ribosome scanning from the capped mRNA 5' end, under the control of initiation factors. However, several cellular mRNAs have been found to have IRES structure to mediate the cap-independent translation (van der Velde, et al. (1999) *Int J Biochem Cell Biol.* 31:87-106). Examples are immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) *Nature* 353:90-94), antennapedia mRNA of Drosophilan (Oh et al. (1992) *Gene and Dev* 6:1643-1653), fibroblast growth factor-2 (FGF-2) (Vagner et al. (1995) *Mol Cell Biol* 15:35-44), platelet-derived growth factor B (PDGF-B) (Bernstein et al. (1997) *J Biol Chem* 272:9356-9362), insulin-like growth factor II (Teerink et al. (1995) *Biochim Biophys Acta* 1264:403-408), and the translation initiation factor eIF4G (Gan et al. (1996) *J Biol Chem* 271:623-626). Table 1 depicts the 5'-noncoding region for BiP and PDGF. Recently, vascular endothelial growth factor (VEGF) was also found to have IRES element (Stein et al. (1998) *Mol Cell Biol* 18:3112-3119; Huez et al. (1998) *Mol Cell Biol* 18:6178-6190).

Apart from the oligopyrimidine tract, nucleotide sequence *per se* does not appear to be important for IRES function. Without wishing to be bound by theory, a possible explanation for the function of an IRES is that it forms secondary and/or tertiary structures which orient particular single-stranded regions of its sequence in a three-dimensional configuration that is conducive to interaction with a mammalian ribosome (either ribosomal protein and/or ribosomal RNA components) and/or initiation factor(s) and/or RNA binding proteins which interact with ribosomes and/or initiation factors. It is also possible that the three-dimensional structure of the IRES

is determined or stabilized by one or more RNA-binding proteins. Thus it is possible to devise synthetic IRES sequences having similar single-stranded regions in a similar three-dimensional configuration.

In certain cases, one or more *trans*-acting cellular proteins may be required for IRES function. For example, the HAV and entero/rhinovirus IRESes function inefficiently *in vitro* in reticulocyte lysates. Supplementation of a reticulocyte lysate with a cytoplasmic extract from HeLa, Krebs II ascites, or L-cells restores activity of entero/rhinovirus IRESes. *See*, for example, Brown *et al.* (1979) *Virology* **97:**396-405; and Dorner *et al.* (1984) *J. Virol.* **50:**507-514. Activity of the HAV IRES *in vitro* is stimulated by liver cytoplasmic extracts. Glass *et al.* (1993) *Virology* **193:**1047-1050. These observations indicate that cell-specific translational regulation can be achieved through the use of a cell-specific IRES. Furthermore, coordinated cell-specific transcriptional and translational regulatory elements can be included in a vector to further increase cell specificity of viral replication. For example, the combination of an AFP-TRE and a HAV-IRES can be used to direct preferential replication of a vector in hepatic cells. Thus, in one illustrative embodiment, a vector comprises an AFP-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In another illustrative embodiment, the vector comprises a probasin-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In yet another illustrative embodiment, a vector comprises a CMV-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

Examples of IRES which can be used in the present invention include those provided in Table 1 and Table 2. An IRES sequence which may be used in the present invention may be tested as follows. A test vector is produced having a reporter gene, such as luciferase, for example, placed under translational control of an IRES to be tested. A desired cell type is transfected with the vector containing the

desired IRES-reporter gene and an assay is performed to detect the presence of the reporter gene. In one illustrative example, the test vector comprises a co-transcribed chloramphenicol transferase (CAT) and luciferase encoding gene transcriptionally driven by a CMV promoter wherein the luciferase encoding gene is translationally driven by an IRES to be tested. Host cells are transiently transfected with the test vector by means known to those of skill in the art and assayed for the presence of luciferase.

IRES may be prepared using standard recombinant and synthetic methods known in the art, and as described in the Examples. For cloning convenience, restriction sites may be engineered into the ends of the IRES fragments to be used.

Transcriptional response elements (TREs)

The adenovirus vectors of the invention comprise target cell specific TREs which direct preferential expression of an operatively linked gene (or genes) in a particular target cell. A TRE can be tissue-specific, tumor-specific, developmental stage-specific, cell status specific, *etc.*, depending on the type of cell present in the tissue or tumor.

Cell- and tissue-specific transcriptional regulatory elements, as well as methods for their identification, isolation, characterization, genetic manipulation and use for regulation of operatively linked coding sequences, are well known in the art. A TRE can be derived from the transcriptional regulatory sequences of a single gene, or sequences from different genes can be combined to produce a functional TRE. A cell-specific TRE is preferentially functional in a limited population (or type) of cells, *e.g.*, prostate cells or liver cells. Accordingly, in some embodiments, the TRE used is preferentially functional in any of the following cell types: prostate; liver; breast; urothelial cells (bladder); colon; lung; ovarian; pancreas; stomach; and uterine. In other embodiments, in accordance with cell status, the TRE is functional in or during: low oxygen conditions (hypoxia); certain stages of cell cycle, such as S phase; elevated temperature; ionizing radiation.

As is known in the art, activity of TREs can be inducible. Inducible TREs generally exhibit low activity in the absence of inducer, and are up-regulated in the presence of inducer. Inducers include, for example, nucleic acids, polypeptides, small molecules, organic compounds and/or environmental conditions such as temperature, pressure or hypoxia. Inducible TREs may be preferred when expression is desired only at certain times or at certain locations, or when it is desirable to titrate the level of expression using an inducing agent. For example, transcriptional activity from the *PSE*-TRE, *PB*-TRE and *hKLK2*-TRE is inducible by androgen, as described herein and in PCT/US98/04080. Accordingly, in one embodiment of the present invention, an adenovirus vector comprises an inducible heterologous TRE.

TRE multimers are also useful in the disclosed vectors. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five promoter fragments. Alternatively, a TRE can comprise one or more promoter regions along with one or more enhancer regions. TRE multimers can also comprise promoter and/or enhancer sequences from different genes. The promoter and enhancer components of a TRE can be in any orientation with respect to each other and can be in any orientation and/or any distance from the coding sequence of interest, as long as the desired cell-specific transcriptional activity is obtained.

The disclosed vectors are designed such that replication is preferentially enhanced in target cells in which the TRE(s) is (are) functional. More than one TRE can be present in a vector, as long as the TREs are functional in the same target cell. However, it is important to note that a given TRE can be functional in more than one type of target cell. For example, the CEA-TRE functions in, among other cell types, gastric cancer cells, colorectal cancer cells, pancreatic cancer cells and lung cancer cells.

A TRE for use in the present vectors may or may not comprise a silencer. The presence of a silencer (*i.e.*, a negative regulatory element known in the art) can assist in shutting off transcription (and thus replication) in non-target cells. Thus,

presence of a silencer can confer enhanced cell-specific vector replication by more effectively preventing replication in non-target cells. Alternatively, lack of a silencer may stimulate replication in target cells, thus conferring enhanced target cell-specificity.

As is readily appreciated by one skilled in the art, a TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily-available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite cell-specific transcription regulatory function. Hence, functionally preserved variants of TREs, comprising nucleic acid substitutions, additions, and/or deletions, can be used in the vectors disclosed herein. Accordingly, variant TREs retain function in the target cell but need not exhibit maximal function. In fact, maximal transcriptional activation activity of a TRE may not always be necessary to achieve a desired result, and the level of induction afforded by a fragment of a TRE may be sufficient for certain applications. For example, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be sufficient if, for example, the target cells are not especially virulent and/or the extent of disease is relatively confined.

Certain base modifications may result in enhanced expression levels and/or cell-specificity. For example, nucleic acid sequence deletions or additions within a TRE can move transcription regulatory protein binding sites closer or farther away from each other than they exist in their normal configuration, or rotate them so they are on opposite sides of the DNA helix, thereby altering spatial relationship among TRE-bound transcription factors, resulting in a decrease or increase in transcription, as is known in the art. Thus, while not wishing to be bound by theory, the present disclosure contemplates the possibility that certain modifications of a TRE will result in modulated expression levels as directed by the TRE, including enhanced cell-

specificity. Achievement of enhanced expression levels may be especially desirable in the case of more aggressive forms of neoplastic growth, and/or when a more rapid and/or aggressive pattern of cell killing is warranted (for example, in an immunocompromised individual).

Transcriptional activity directed by a TRE (including both inhibition and enhancement) can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA and/or of a protein product encoded by the sequence under control of (*i.e.*, operably linked to) a TRE.

As discussed herein, a TRE can be of varying lengths, and of varying sequence composition. The size of a heterologous TRE will be determined in part by the capacity of the viral vector, which in turn depends upon the contemplated form of the vector (see *infra*). Generally minimal sizes are preferred for TREs, as this provides potential room for insertion of other sequences which may be desirable, such as transgenes (discussed *infra*) and/or additional regulatory sequences. In a preferred embodiment, such an additional regulatory sequence is an IRES. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, larger TRE sequences can be used as long as the resultant adenoviral vector remains replication-competent.

An adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb, without requiring deletion of viral sequences. If non-essential sequences are removed from the adenovirus genome, an additional 4.6 kb of insert can be tolerated (*i.e.*, for a total insertion capacity of about 6.4 kb). Examples of non-essential adenoviral sequences that can be deleted are E3, and E4 sequences other than those which encode E4 ORF6.

To minimize non-specific replication, endogenous (*e. g.*, adenovirus) TREs are preferably removed from the vector. Besides facilitating target cell-specific

replication, removal of endogenous TREs also provides greater insert capacity in a vector, which may be of special concern if an adenoviral vector is to be packaged within a virus particle. Even more importantly, deletion of endogenous TREs prevents the possibility of a recombination event whereby a heterologous TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication). In one embodiment, an adenoviral vector is constructed such that the endogenous transcription control sequences of adenoviral genes are deleted and replaced by one or more heterologous TREs. However, endogenous TREs can be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments are constructed by inserting heterologous TREs between an endogenous TRE and a replication gene coding segment. Requisite cell-specific replication preference is determined by conducting assays that compare replication of the adenovirus vector in a cell which allows function of the heterologous TREs with replication in a cell which does not.

Generally, a TRE will increase replication of a vector in a target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500- fold, even more preferably at least about 1000-fold, compared to basal levels of replication in the absence of a TRE. The acceptable differential can be determined empirically (by measurement of mRNA levels using, for example, RNA blot assays, RNase protection assays or other assays known in the art) and will depend upon the anticipated use of the vector and/or the desired result.

Replication-competent adenovirus vectors directed at specific target cells can be generated using TREs that are preferentially functional in a target cell. In one embodiment of the present invention, the target cell is a tumor cell. Non-limiting

examples of tumor cell-specific heterologous TREs, and their respective target cells, include: probasin (PB), target cell, prostate cancer (PCT/US98/04132); α-fetoprotein (*AFP*), target cell liver cancer (PCT/US98/04084); mucin-like glycoprotein DF3 (*MUC1*), target cell, breast carcinoma (PCT/US98/04080); carcinoembryonic antigen (*CEA*), target cells, colorectal, gastric, pancreatic, breast, and lung cancers (PCT/US98/04133); plasminogen activator urokinase (*uPA*) and its receptor gene, target cells, breast, colon, and liver cancers (PCT/US98/04080); *E2F1* (cell cycle S-phase specific promoter); target cell, tumors with disrupted retinoblastoma gene function, and *HER-2/neu* (*c-erbB2/neu*), target cell, breast, ovarian, stomach, and lung cancers (PCT/US98/04080); tyrosinase, target cell, melanoma cells as described herein and uroplakins, target cell, bladder cells as described herein. Methods for identification, isolation, characterization and utilization of additional target cell-specific TREs are readily available to those of skill in the art.

In addition, tumor-specific TREs can be used in conjunction with tissue-specific TREs from the following exemplary genes (tissue in which the TREs are specifically functional are in parentheses): hypoxia responsive element, vascular endothelial growth factor receptor (endothelium), albumin (liver), factor VII (liver), fatty acid synthase (liver), Von Willebrand factor (brain endothelium), alpha-actin and myosin heavy chain (both in smooth muscle), synthetase I (small intestine) Na^+-K^+-Cl^- transporter (kidney). Additional tissue-specific TREs are known in the art.

In one embodiment of the present invention, a target cell-specific, heterologous TRE is tumor cell-specific. A vector can comprise a single tumor cell-specific TRE or multiple heterologous TREs which are tumor cell-specific and functional in the same cell. In another embodiment, a vector comprises one or more heterologous TREs which are tumor cell-specific and additionally comprises one or more heterologous TREs which are tissue specific, whereby all TREs are functional in the same cell.

Prostate-specific TREs

In one embodiment, adenovirus vectors comprise heterologous TREs that are prostate cell specific. For example, TREs that function preferentially in prostate cells and can be used to target adenovirus replication to prostate neoplasia, include, but are not limited to, TREs derived from the prostate-specific antigen gene (*PSA*-TRE) (Henderson U.S. Patent No. 5,698,443); the glandular kallikrein-1 gene (from the human gene, *hKLK2*-TRE) (PCT US98/16312), and the probasin gene (*PB*-TRE) (PCT/US98/04132). All three of these genes are preferentially expressed in prostate cells and their expression is androgen-inducible. Generally, expression of genes responsive to androgen induction is mediated by an androgen receptor (AR).

Prostate-specific Antigen (PSA)

PSA is synthesized exclusively in prostatic epithelial cells and is synthesized in these cells whether they are normal, hyperplastic, or malignant. This tissue-specific expression of PSA has made it an excellent biomarker for benign prostatic hyperplasia (BPH) and prostatic carcinoma (CaP). Normal serum levels of PSA are typically below 5 ng/ml, with elevated levels indicative of BPH or CaP. Lundwall *et al.* (1987) *FEBS Lett.* **214:**317; Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161:**1151; and Riegmann *et al.* (1991) *Molec. Endocrin.* **5:**1921.

The region of the *PSA* gene that provides androgen-dependent cell specificity, particularly in prostate cells, involves approximately 6.0 kilobases (kb). Schuur *et al.* (1996) *J. Biol. Chem.* **271:**7043-7051. An enhancer region of approximately 1.5 kb in humans is located between nt -5322 and nt -3739, relative to the transcription start site of the *PSA* gene. Within these enhancer sequences is an androgen response element (ARE) a sequence which binds androgen receptor. The sequence coordinates of the *PSA* promoter are from about nt -540 to nt +8 relative to the transcription start site. Juxtapositioning of the enhancer and promoter yields a fully functional, minimal prostate-specific TRE (*PSA*-TRE). Other portions of this approximately 6.0 kb

region of the *PSA* gene can be used in the vectors described herein, as long as requisite functionality is maintained.

Human glandular Kallikrein (hKLK2)

Human glandular kallikrein (*hKLK2*, encoding the hK2 protein) is expressed exclusively in the prostate and its expression is up-regulated by androgens, primarily through transcriptional activation. Wolf *et al.* (1992) *Molec. Endocrinol.* **6:**753-762; Morris (1989) *Clin. Exp. Pharm. Physiol.* **16:**345-351; Qui *et al.* (1990) *J. Urol.* **144:**1550-1556; and Young *et al.* (1992) *Biochem.* **31:**818-824. The levels of hK2 found in various tumors and in the serum of patients with prostate cancer indicate that hK2 antigen may be a significant marker for prostate cancer. Charlesworth *et al.* (1997) *Urology* **49:**487-493. Expression of hK2 has been detected in each of 257 radical prostatectomy specimens analyzed. Darson *et al.* (1997) *Urology* **49:**857-862. The intensity and extent of hK2 expression, detected using specific antibodies, was observed to increase from benign epithelium to high-grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma.

The activity of the *hKLK2* promoter has been described and a region up to nt -2256 relative to the transcription start site was previously disclosed. Schedlich *et al.* (1987) *DNA* **6:**429-437. The *hKLK2* promoter is androgen responsive and, in plasmid constructs wherein the promoter alone controls the expression of a reporter gene, expression of the reporter gene is increased approximately 10-fold in the presence of androgen. Murtha *et al.* (1993) *Biochem.* **32:**6459-6464. *hKLK2* enhancer activity is found within a polynucleotide sequence approximately nt -12,014 to nt -2257 relative to the start of transcription and, when this sequence is operably linked to an *hKLK2* promoter and a reporter gene, transcription of operably-linked sequences in prostate cells increases in the presence of androgen to levels approximately 30-fold to approximately 100-fold greater than the level of transcription in the absence of androgen. This induction is generally independent of the orientation and position of the enhancer sequences. Enhancer activity has also

been demonstrated in the following regions (all relative to the transcription start site): about nt -3993 to about nt -3643, about nt -4814 to about nt -3643, about nt -5155 to about nt -3387, about nt -6038 to about nt -2394.

Thus, a *hKLK2* enhancer can be operably linked to an *hKLK2* promoter or a heterologous promoter to form a *hKLK2* transcriptional regulatory element (*hKLK2*-TRE). A *hKLK2*-TRE can then be operably linked to a heterologous polynucleotide to confer *hKLK2*-TRE-specific transcriptional regulation on the linked gene, thus increasing its expression.

Probasin

The rat probasin (*PB*) gene encodes an androgen and zinc-regulated protein first characterized in the dorsolateral prostate of the rat. Dodd *et al.* (1983) *J. Biol. Chem.* **258:**10731–10737; Matusik *et al.* (1986) *Biochem. Cell. Biol.* **64:**601-607; and Sweetland *et al.* (1988) *Mol. Cell. Biochem.* **84:**3-15. The dorsolateral lobes of the murine prostate are considered the most homologous to the peripheral zone of the human prostate, where approximately 68% of human prostate cancers are thought to originate.

A *PB*-TRE has been shown to exist in an approximately 0.5 kb fragment of sequence upstream of the probasin coding sequence, from about nt -426 to about nt +28 relative to the transcription start site. This minimal promoter sequence from the PB gene appears to provide sufficient information to direct prostate-specific developmental- and hormone -regulated expression of an operably linked heterologous gene in transgenic mice. Greenberg *et al.* (1994) *Mol. Endocrinol.* **8:**230-239.

Alpha-fetoprotein

α-fetoprotein (AFP) is an oncofetal protein, the expression of which is primarily restricted to developing tissues of endodermal origin (yolk sac, fetal liver, and gut), although the level of its expression varies greatly depending on the tissue and the developmental stage. AFP is of clinical interest because the serum

concentration of AFP is elevated in a majority of hepatoma patients, with high levels of AFP found in patients with advanced disease. High serum AFP levels in patients appear to be due to AFP expression in hepatocellular carcinoma (HCC), but not in surrounding normal liver. Thus, expression of the AFP gene appears to be characteristic of hepatoma cells. An AFP-TRE is described in for example PCT/US98/04084.

According to published reports, the AFP-TRE is responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with AFP-producing cells, such as AFP-binding protein (see, for example, U.S. Pat. No. 5,302,698) and comprises at least a portion of an AFP promoter and/or an AFP enhancer. Cell-specific TREs from the *AFP* gene have been identified. For example, the cloning and characterization of human AFP-specific enhancer activity is described in Watanabe *et al.* (1987) *J. Biol. Chem.* **262:**4812-4818. A 5' *AFP* regulatory region (containing the promoter, putative silencer, and enhancer) is contained within approximately 5 kb upstream from the transcription start site.

Within the AFP regulatory region, a human *AFP* enhancer region is located between about nt -3954 and about nt -3335, relative to the transcription start site of the *AFP* gene. The human *AFP* promoter encompasses a region from about nt -174 to about nt +29. Juxtapositioning of these two genetic elements, yields a fully functional *AFP*-TRE. Ido *et al.* (1995) *Cancer Res.* **55:**3105-3109 describe a 259 bp promoter fragment (nt -230 to nt +29) that is specific for expression in HCC cells. The *AFP* enhancer, located between nt -3954 and nt -3335 relative to the transcription start site, contains two regions, denoted A and B. The promoter region contains typical TATA and CAAT boxes. Preferably, the *AFP*-TRE contains at least one enhancer region. More preferably, the *AFP*-TRE contains both enhancer regions.

Suitable target cells for vectors containing *AFP*-TREs are any cell type that allow an *AFP*-TRE to function. Preferred are cells that express or produce AFP, including, but not limited to, tumor cells expressing AFP. Examples of such cells are

hepatocellular carcinoma (HCC) cells, gonadal and other germ cell tumors (especially endodermal sinus tumors), brain tumor cells, ovarian tumor cells, acinar cell carcinoma of the pancreas (Kawamoto *et al.* (1992) *Hepatogastroenterology* **39:**282–286), primary gall bladder tumor (Katsuragi *et al.* (1989) *Rinsko Hoshasen* **34:**371–374), uterine endometrial adenocarcinoma cells (Koyama *et al.* (1996) *Jpn. J. Cancer Res.* **87:**612-617), and any metastases of the foregoing (which can occur in lung, adrenal gland, bone marrow, and/or spleen). In some cases, metastatic disease to the liver from certain pancreatic and stomach cancers produce AFP. Especially preferred as target cells for an AFP-TRE are hepatocellular carcinoma cells and any of their metastases.

AFP production can be measured (and hence AFP-producing cells can be identified) using immunoassays standard in the art, such as RIA, ELISA or protein immunoblotting (Western blots) to determine levels of AFP protein production; and/or RNA blotting (Northern blots) to determine AFP mRNA levels. Alternatively, such cells can be identified and/or characterized by their ability to activate transcriptionally an *AFP*-TRE (*i.e.*, allow an *AFP*-TRE to function).

See also co-owned PCT W098/39465 regarding AFP-TREs. As described in more detail therein, an AFP-TRE can comprise any number of configurations, including, but not limited to, an AFP promoter; an AFP enhancer; an AFP promoter and an AFP enhancer; an AFP promoter and a heterologous enhancer; a heterologous promoter and an AFP enhancer; and multimers of the foregoing. The promoter and enhancer components of an AFP-TRE can be in any orientation and/or distance from the coding sequence of interest, as long as the desired AFP cell-specific transcriptional activity is obtained. An adenovirus vector of the present invention can comprise an AFP-TRE endogenous silencer element or the AFP-TRE endogenous silencer element can be deleted.

Urokinase Plasminogen Activator

The protein urokinase plasminogen activator (uPA) and its cell surface receptor, urokinase plasminogen activator receptor (uPAR), are expressed in many of the most frequently-occurring neoplasms and appear to represent important proteins in cancer metastasis. Both proteins are implicated in breast, colon, prostate, liver, renal, lung and ovarian cancer. Sequence elements that regulate uPA and uPAR transcription have been extensively studied. Riccio *et al.* (1985) *Nucleic Acids Res.* **13**:2759-2771; Cannio *et al.* (1991) *Nucleic Acids Res.* **19**:2303–2308.

Carcinoembryonic antigen (CEA)

CEA is a 180,000 Dalton, tumor-associated, glycoprotein antigen present on endodermally-derived neoplasms of the gastrointestinal tract, such as colorectal, gastric (stomach) and pancreatic cancer, as well as other adenocarcinomas such as breast and lung cancers. CEA is of clinical interest because circulating CEA can be detected in the great majority of patients with CEA-positive tumors. In lung cancer, about 50% of total cases have circulating CEA, with high concentrations of CEA (greater than 20 ng/ml) often detected in adenocarcinomas. Approximately 50% of patients with gastric carcinoma are serologically positive for CEA.

The 5'-flanking sequence of the *CEA* gene has been shown to confer cell-specific activity. The *CEA* promoter region, approximately the first 424 nucleotides upstream of the transcriptional start site in the 5' flanking region of the gene, was shown to confer cell-specific activity by virtue of providing higher promoter activity in CEA-producing cells than in non-producing HeLa cells. Schrewe *et al.* (1990) *Mol. Cell. Biol.* **10**:2738-2748. In addition, cell-specific enhancer regions have been found. *See* PCT/GB/02546 The *CEA* promoter, putative silencer, and enhancer elements appears to be contained within a region that extends approximately 14.5 kb upstream from the transcription start site. Richards et al. (1995); PCT/GB/02546. Further characterization of the 5'-flanking region of the *CEA* gene by Richards *et al.* (1995) *supra* indicated that two upstream regions (one between about -13.6 and

about -10.7 kb, and the other between about -6.1 and about-4.0 kb), when linked to the multimerized promoter, resulted in high-level and selective expression of a reporter construct in CEA-producing LoVo and SW1463 cells. Richards et al. (1995) *supra* also localized the promoter region between about nt -90 and about nt +69 relative to the transcriptional start site, with the region between about nt -41 and about nt -18 being essential for expression. PCT/GB/02546 describes a series of 5'-flanking *CEA* fragments which confer cell-specific activity, including fragments comprising the following sequences: about nt -299 to about nt +69; about nt -90 to about nt +69; nt -14,500 to nt -10,600; nt -13,600 to nt -10,600; and nt -6100 to nt -3800, with all coordinates being relative to the transcriptional start point. In addition, cell-specific transcription activity is conferred on an operably linked gene by the *CEA* fragment from nt -402 to nt +69.

CEA-TREs for use in the vectors disclosed herein are derived from mammalian cells, including, but not limited to, human cells. Thus, any of the *CEA*-TREs can be used as long as the requisite desired functionality is displayed by the vector.

Mucin

The protein product of the *MUC1* gene (known as mucin, MUC1 protein; episialin; polymorphic epithelial mucin or PEM; EMA; DF3 antigen; NPGP; PAS-O; or CA15.3 antigen) is normally expressed mainly at the apical surface of epithelial cells lining the glands or ducts of the stomach, pancreas, lungs, trachea, kidney, uterus, salivary glands, and mammary glands. Zotter *et al.* (1988) *Cancer Rev.* **11–12:**55–101; and Girling *et al.* (1989) *Int. J. Cancer* **43:**1072–1076. However, mucin is overexpressed in 75–90% of human breast carcinomas. Kufe *et al.* (1984) *Hybridoma* **3:**223–232. For reviews, see Hilkens (1988) *Cancer Rev.* **11–12:**25–54; and Taylor-Papadimitriou, *et al.* (1990) *J. Nucl. Med. Allied Sci.* **34:**144–150. Mucin protein expression correlates with the degree of breast tumor differentiation. Lundy *et al.* (1985) *Breast Cancer Res. Treat.* **5:**269–276.

Overexpression of the *MUC1* gene in human breast carcinoma cells MCF-7 and ZR-75-1 appears to occur at the transcriptional level. Kufe *et al.* (1984) *supra*; Kovarik (1993) *J. Biol. Chem.* **268:**9917–9926; and Abe *et al.* (1990) *J. Cell. Physiol.* **143:**226–231. The regulatory sequences of the *MUC1* gene have been cloned, including the approximately 0.9 kb upstream of the transcription start site which contains a TRE that appears to be involved in cell-specific transcription. Abe *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90:**282-286; Kovarik et al. (1993) *supra*; and Kovarik *et al.* (1996) *J. Biol. Chem.* **271:**18140–18147.

MUC1-TREs are derived from mammalian cells, including but not limited to, human cells. Preferably, the *MUC1*-TRE is human. In one embodiment, the *MUC1*-TRE contains the entire 0.9 kb 5' flanking sequence of the *MUC1* gene. In other embodiments, *MUC1*-TREs comprise the following sequences (relative to the transcription start site of the *MUC1* gene) operably-linked to a promoter: about nt -725 to about nt +31, about nt -743 to about nt +33, about nt -750 to about nt +33, and about nt -598 to about nt +485.

c-erbB2/*HER-2*/neu

The *c-erbB2/neu* gene (*HER-2/neu* or *HER*) is a transforming gene that encodes a 185 kD epidermal growth factor receptor-related transmembrane glycoprotein. In humans, the c-erbB2/neu protein is expressed during fetal development and, in adults, the protein is weakly detectable (by immunohistochemistry) in the epithelium of many normal tissues. Amplification and/or over-expression of the *c-erbB2/neu* gene has been associated with many human cancers, including breast, ovarian, uterine, prostate, stomach and lung cancers. The clinical consequences of overexpression of the c-erbB2/neu protein have been best studied in breast and ovarian cancer. c-erbB2/neu protein over-expression occurs in 20 to 40% of intraductal carcinomas of the breast and 30% of ovarian cancers, and is associated with a poor prognosis in subcategories of both diseases.

Human, rat and mouse *c-erbB2/neu* TREs have been identified and shown to confer transcriptional activity specific to *c-erbB2/neu*-expressing cells. Tal *et al.* (1987) *Mol. Cell. Biol.* **7:**2597–2601; Hudson *et al.* (1990) *J. Biol. Chem.* **265:**4389–4393; Grooteclaes *et al.* (1994) *Cancer Res.* **54:**4193–4199; Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. USA* **84:**4374–4378; and Scott *et al.* (1994) *J. Biol. Chem.* **269:**19848–19858.

Melanocyte-specific TRE

It has been shown that some genes which encode melanoma proteins are frequently expressed in melanoma/melanocytes, but silent in the majority of normal tissues. A variety of melanocyte-specific TRE are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with melanocytes, and comprise at least a portion of a melanocyte-specific promoter and/or a melanocyte-specific enhancer. Known transcription factors that control expression of one or more melanocyte-specific genes include the microphthalmia associated transcription factor MITF. Yasumoto et al. (1997) *J. Biol. Chem.* 272:503-509. Other transcription factors that control expression of one or more melanocyte specific genes include MART-1/Melan-A, gp100, TRP-1 and TRP-2

Methods are described herein for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

In some embodiments, the melanocyte-specific TREs used in this invention are derived from mammalian cells, including but not limited to, human, rat, and mouse. Any melanocyte-specific TREs may be used in the adenoviral vectors of the invention. Rodent and human 5' flanking sequences from genes expressed specifically or preferentially in melanoma cells have been described in the literature and are thus made available for practice of this invention and need not be described in detail herein. The following are some examples of melanocyte-specific TREs which can be used. A promoter and other control elements in the human tyrosinase gene 5'

flanking region have been described and sequences have been deposited as GenBank Accession Nos. X16073 and D10751. Kikuchi et al. (1989) *Biochim. Biophys. Acta* 1009:283-286; and Shibata et al. (1992) *J. Biol. Chem.* 267:20584-20588. A cis-acting element has been defined that enhances melanocyte-specific expression of human tyrosinase gene. This element comprises a 20-bp sequence known as tyrosinase distal element (TDE), contains a CATGTG motif, and lies at positions about -1874 to about -1835 relative to the human tyrosinase gene transcription start site. Yasumoto et al. (1994) *Mol. Cell. Biol.* 14:8058-8070. A promoter region comprising sequences from about -209 to +61 of the human tyrosinase gene was found to direct melanocyte-specific expression. Shibata (1992). Similarly, the mouse tyrosinase 5' flanking region has been analyzed and a sequence deposited as GenBank Accession Nos. D00439 and X51743. Klüppel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:3777-3788. A minimal promoter has been identified for the mouse TRP-1 gene, and was reported to encompass nucleotides -44 to +107 relative to the transcription start site. Lowings et al. (1992) *Mol. Cell. Biol.* 12:3653-3662. Two regulatory regions required for melanocyte-specific expression of the human TRP-2 gene have been identified. Yokoyama et al. (1994) *J. Biol. Chem.* 269:27080-27087. A human MART-1 promoter region has been described and deposited as GenBank Accession No. U55231. Melanocyte-specific promoter activity was found in a 233-bp fragment of the human MART-1 gene 5' flanking region. Butterfield et al. (1997) *Gene* 191:129-134. A basic-helix-loop-helix/leucine zipper-containing transcription factor, MITF (microphthalmia associated transcription factor) was reported to be involved in transcriptional activation of tyrosinase and TRP-1 genes. Yasumoto et al. (1997) *J. Biol. Chem.* 272:503-509.

In some embodiments, a melanocyte-specific TRE comprises sequences derived from the 5' flanking region of a human tyrosinase gene depicted in Table 3. In some of these embodiments, the melanocyte-specific TRE comprises tyrosinase nucleotides from about -231 to about +65 relative to the transcription start site (from

about nucleotide 244 to about nucleotide 546 of SEQ ID NO:10) and may further comprise nucleotides from about -1956 to about -1716 relative to the human tyrosinase transcription start site (from about nucleotide 6 to about nucleotide 243 of SEQ ID NO:10). A melanocyte-specific TRE can comprise nucleotides from about --231 to about + 65 juxtaposed to nucleotides from about -1956 to about -1716. It has been reported that nucleotides from about -1956 to about -1716 relative to the human tyrosinase transcription start site can confer melanocyte-specific expression of an operably linked reporter gene with either a homologous or a heterologous promoter. Accordingly, in some embodiments, a melanocyte-specific TRE comprises nucleotides from about -1956 to about -1716 operably linked to a heterologous promoter.

A melanocyte-specific TRE can also comprise multimers. For example, a melanocyte-specific TRE can comprise a tandem series of at least two, at least three, at least four, or at least five tyrosinase promoter fragments. Alternatively, a melanocyte-specific TRE could have one or more tyrosinase promoter regions along with one or more tyrosinase enhancer regions. These multimers may also contain heterologous promoter and/or enhancer sequences.

Cell status-specific TREs

Cell status-specific TREs for use in the adenoviral vectors of the present invention can be derived from any species, preferably a mammal. A number of genes have been described which are expressed in response to, or in association with, a cell status. Any of these cell status-associated genes may be used to generate a cell status-specific TRE.

An example of a cell status is cell cycle. An exemplary gene whose expression is associated with cell cycle is E2F-1, a ubiquitously expressed, growth-regulated gene, which exhibits peak transcriptional activity in S phase. Johnson et al. (1994) *Genes Dev.* 8:1514-1525. The RB protein, as well as other members of the RB family, form specific complexes with E2F-1, thereby inhibiting its ability to

activate transcription. Thus, E2F-1-responsive promoters are down-regulated by RB. Many tumor cells have disrupted RB function, which can lead to de-repression of E2F-1-responsive promoters, and, in turn, de-regulated cell division.

Accordingly, in one embodiment, the invention provides an E3-containing adenoviral vector in which an adenoviral gene (preferably a gene necessary for replication) is under transcriptional control of a cell status-specific TRE, wherein the cell status-specific TRE comprises a cell cycle-activated TRE. In one embodiment, the cell cycle-activated TRE is an E2F1 TRE.

Another group of genes that are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335. An HRE from a rat enolase-1 promoter is depicted in Table 3.

Accordingly, in one embodiment, an adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE. In one embodiment, the cell status-specific TRE comprises the HRE depicted in Table 3.

Other cell status-specific TREs include heat-inducible (i.e., heat shock) promoters, and promoters responsive to radiation exposure, including ionizing radiation and UV radiation. For example, the promoter region of the early growth response-1 (Egr-1) gene contains an element(s) inducible by ionizing radiation. Hallahan et al. (1995) *Nat. Med.* 1:786-791; and Tsai-Morris et al. (1988) *Nucl. Acids. Res.* 16:8835-8846. Heat-inducible promoters, including heat-inducible elements, have been described. See, for example Welsh (1990) in "Stress Proteins in Biology and Medicine", Morimoto, Tisseres, and Georgopoulos, eds. Cold Spring Harbor Laboratory Press; and Perisic et al. (1989) *Cell* 59:797-806. Accordingly, in some embodiments, the cell status-specific TRE comprises an element(s) responsive to ionizing radiation. In one embodiment, this TRE comprises a 5' flanking sequence of an Egr-1 gene. In other embodiments, the cell status-specific TRE comprises a heat shock responsive element.

The cell status-specific TREs listed above are provided as non-limiting examples of TREs that would function in the instant invention. Additional cell status-specific TREs are known in the art, as are methods to identify and test cell status specificity of suspected cell status-specific TREs.

Urothelial cell-specific TREs

Any urothelial cell-specific TRE may be used in the adenoviral vectors of the invention. A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am.*

J. Pathol. 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683).

Preferred urothelial cell-specific TREs include TREs derived from the uroplakins UPIa, UPIb, UPII, and UPIII, as well as urohingin. A uroplakin TRE may be from any species, depending on the intended use of the adenovirus, as well as the requisite functionality is exhibited in the target or host cell. Significantly, adenovirus constructs comprising a urothelial cell-specific TREs have observed that such constructs are capable of selectively replicating in urothelial cells as opposed to smooth muscle cells, which adjoin urothelial cells in the bladder.

Uroplakin

Urothelial-specific TREs derived from the *hUPII* gene are described herein. Accordingly, in some embodiments, an adenovirus vector of the invention comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 2.2 kb sequence from the 5' flanking region of hUPII gene, as shown in Table 3. In other embodiments, an adenovirus vector of the invention comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises a 1.8 kb sequence from the 5' flanking region of hUPII gene, from nucleotides 430 to 2239 as shown in Table 3. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 2.2 kb sequence depicted in Table 3, or a functional portion of the 1.8 kb sequence of nucleotides 430 to 2239 of the sequence depicted in Table 3, such as a fragment of 2000 bp or less, 1500 bp or less, or 1000 bp or less, 600 bp less, or at least 200 bp which includes the 200 bp fragment of the hUPII 5'-flanking region.

A 3.6 kb 5'-flanking sequence located from the mouse UPII (mUPII) gene which confers urothelial cell-specific transcription on heterologous genes is one urothelial cell-specific TRE useful in vectors of the instant invention (Table 3). Smaller TREs (*i.e.*, 3500 bp or less, more preferably less than about 2000 bp, 1500 bp, or 1000 bp) are preferred. Smaller TREs derived from the mUPII 3.6 kb fragment are one group of preferred urothelial cell-specific TREs. In particular, Inventors have identified an approximately 600 bp fragment from the 5' flanking DNA of the mUPII gene, which contains 540 bp of 5' untranslated region (UTR) of the mUPII gene, that confers urothelial cell-specific expression on heterologous genes.

Accordingly, in some embodiments, an adenovirus vector of the invention comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 3.6 kb sequence from the 5' flanking region of mouse UPII gene, as shown in Table 3. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 3.6 kb sequence depicted in Table 3, such as a fragment of 3500 bp or less, 2000 bp or less, 1500 bp or less, or 1000 bp or less which includes the 540 bp fragment of 5' UTR. The urothelial cell-specific TRE may also be a sequence which is substantially identical to the 3.6 kb mUPII 5'-flanking region or any of the described fragments thereof.

As an example of how urothelial cell-specific TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), a green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are

transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative target cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection) and DEAE dextran. Suitable host cells include any urothelial cell type, including but not limited to, KU-1, MYP3 (a non-tumorigenic rat urothelial cell line), 804G (rat bladder carcinoma cell line), cultured human urothelial cells (HUC), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. Non-urothelial cells, such as LNCaP, HBL-100, HLF, HLE, 3T3, Hep3B, HuH7, CADO-LC9, and HeLa are used as a control. Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between urothelial cells and control indicates presence or absence of transcriptional activation.

Comparisons between or among various urothelial cell-specific TREs can be assessed by measuring and comparing levels of expression within a single urothelial cell line. It is understood that absolute transcriptional activity of a urothelial cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the urothelial cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

Alternatively a putative urothelial cell-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells that allow a urothelial cell-specific TRE to function. For this assay, constructs containing an adenovirus gene essential to replication operatively linked to a putative urothelial cell-specific TRE

are transfected into urothelial cells. Viral replication in those cells is compared, for example, to viral replication by wild type adenovirus in those cells and/or viral replication by the construct in non-urothelial cells.

TRE configurations

A TRE as used in the present invention can be present in a variety of configurations. A TRE can comprise multimers. For example, a TRE can comprise a tandem series of at least two, at least three, at least four, or at least five target cell-specific TREs. These multimers may also contain heterologous promoter and/or enhancer sequences.

Optionally, a transcriptional terminator or transcriptional "silencer" can be placed upstream of the target cell-specific TRE, thus preventing unwanted read-through transcription of the coding segment under transcriptional control of the target cell-specific TRE. Also, optionally, the endogenous promoter of the coding segment to be placed under transcriptional control of the target cell-specific TRE can be deleted.

A target cell-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., a non-target cell). Thus, presence of a silencer may confer enhanced target cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced target cell-specific replication due to more effective replication in target cells.

It is also understood that the invention includes a target cell-specific TRE regulating the transcription of a bicistronic mRNA in which translation of the second mRNA is associated by an IRES. An adenovirus vector may further include an additional heterologous TRE which may or may not be operably linked to the same gene(s) as the target cell-specific TRE. For example a TRE (such as a cell type-specific or cell status-specific TRE) may be juxtaposed to a second type of target-

cell-specific TRE. "Juxtaposed" means a target cell-specific TRE and a second TRE transcriptionally control the same gene. For these embodiments, the target cell-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene.

As is readily appreciated by one skilled in the art, a target cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite target cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the TRE nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. The variants of the sequences disclosed herein may be 80%, 85%, 90%, 95%, 98%, 99% or more identical, as measured by, for example, ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using efault parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2 to any of the urothelial cell-specific TRE sequences disclosed herein. Variants of target cell-specific TRE sequences may also hybridize at high stringency, that is at 68°C and 0.1X SSC, to any of the target cell-specific TRE sequences disclosed herein.

In terms of hybridization conditions, the higher the sequence identity required, the more stringent are the hybridization conditions if such sequences are determined by their ability to hybridize to a sequence of TRE disclosed herein. Accordingly, the invention also includes polynucleotides that are able to hybridize to a sequence comprising at least about 15 contiguous nucleotides (or more, such as about 25, 35, 50, 75 or 100 contiguous nucleotides) of a TRE disclosed herein. The hybridization conditions would be stringent, i.e., 80°C (or higher temperature) and 6M SSC (or less

concentrated SSC). Another set of stringent hybridization conditions is 68°C and 0.1 X SSC. For discussion regarding hybridization reactions, see below.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989) at page 7.52. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water. An exemplary set of stringent hybridization conditions is 68°C and 0.1 X SSC.

"T_m" is the temperature in degrees Celcius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-Crick base pairing dissociates into single strands under conditions of the experiment. T_m may be predicted according to a standard formula, such as:

$$T_m = 81.5 + 16.6 \log[X^+] + 0.41\ (\%G/C) - 0.61\ (\%F) - 600/L$$

where $[X^+]$ is the cation concentration (usually sodium ion, Na^+) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable

in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

Determination of TRE activity

Activity of a TRE can be determined, for example, as follows. A TRE polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing a promoter (if no promoter element is present in the TRE) and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ* gene), luciferase (encoded by the *luc* gene), alkaline phosphatase (AP), green fluorescent protein (GFP), and horseradish peroxidase (HRP). Such vectors and assays are readily available, from, *inter alia*, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes, DEAE dextran-mediated transfer, particle bombardment or direct injection. TRE activity is measured by detection and/or quantitation of reporter gene-derived mRNA and/or protein. Reporter protein product can be detected directly (*e.g.*, immunochemically) or through its enzymatic activity, if any, using an appropriate substrate. Generally, to determine cell specific activity of a TRE, a TRE-reporter gene construct is introduced into a variety of cell types. The amount of TRE activity is determined in each cell type and compared to that of a reporter gene construct lacking the TRE. A TRE is determined to be cell-specific if it is preferentially functional in one cell type, compared to a different type of cell.

Adenovirus early genes

The adenovirus vectors of the invention comprise two or more genes which are co-transcribed under the control of a target cell-specific TRE wherein the second gene is under translational control of an IRES. One or more of the genes can be an adenovirus gene, preferably an adenovirus gene essential for replication. Any gene that is essential for adenovirus replication, such as E1A, E1B, E2, E4 or any of the late genes, is useful. The adenovirus may also comprise E3. In addition, one or more of the genes can be a transgene or heterologous gene. Any of the various adenovirus serotypes can be used, such as, for example, Ad2, Ad5, Ad12 and Ad40. For purposes of illustration, the Ad5 serotype is exemplified herein.

The E1A gene is expressed immediately (between 0 and 2 hours) after viral infection, before any other viral genes. E1A protein is a *trans*-acting positive transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are also expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* **651**:175–208; Flint (1986) *Advances Virus Research* **31**:169–228; and Grand (1987) *Biochem. J.* **241**:25–38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* **31**:35–81. The transcription start site of Ad5 E1A is at coordinate 498 and the ATG start site of the E1A protein is at coordinate 560 in the virus genome.

The E1B protein is necessary in *trans* for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey *et al.* (1993) *Virology* **193**:631; Bailey *et al.* (1994) *Virology*

202:695-706. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box, and extends from Ad5 nt 1636 to 1701.

Adenovirus E1B 19-kDa (19K) protein is a potent inhibitor of apoptosis and cooperates with E1A to produce oncogenic transformation of primary cells (Rao, et al., 1992, *Cell Biology*, 89:7742-7746). During productive adenovirus infection, E1A stimulates host cell DNA synthesis, thereby causing cells to aberrantly go through the cell cycle. In response to cell cycle deregulation, the host cell undergoes apoptosis. As a defense mechanism, the E1B 19-kDa protein inhibits this E1A-induced apoptosis and allows assembly of viral progeny to be completed before the cell commits suicide. E1B 19-kDa conducts anti-apoptotic function by multiple mechanisms. E1B 19-kDa inhibits the apoptosis of multiple stimuli, including E1a, p53 and TNF, for example. According to wild-type Ad5 , the E1B 19-kDa region is located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA, 89:*7742-7746.

In a preferred embodiment, expression of the E1A and E1B regions of the Ad genome is facilitated in a cell-specific fashion by placing a cell-specific TRE upstream of E1A and a internal ribosome entry site between E1A and E1B.

The E2 region of adenovirus encodes proteins related to replication of the adenoviral genome, including the 72 kD DNA-binding protein, the 80 kD precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

The E2 early promoter of Ad5 is located between nucleotides 27,050 and 27,150, and consists of a major and a minor transcription initiation site (the latter accounting for about 5% of E2 transcripts), two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site. For a

detailed review of E2 promoter architecture see Swaminathan *et al.* (1995) *Curr. Topics in Micro. and Imm.* **199** part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps by only a few base pairs with sequences on the counterstrand which encode a 33 kD protein. Notably, an SpeI restriction site (Ad5 position 27,082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA box from the upstream E2F and ATF binding sites. Therefore, insertion of a heterologous TRE having SpeI ends into the SpeI site disrupts the endogenous E2 early promoter of Ad5 and allows TRE-regulated expression of E2 transcripts.

An E3 region refers to the region of the adenoviral genome that encodes the E3 products. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. Generally, the E3 region is located between about 28583 and about 30470 of the adenoviral genome. An E3 region for use in the present invention may be from any adenovirus serotype. An E3 sequence is a polynucleotide sequence that contains a sequence from an E3 region. In some embodiments, the sequence encodes ADP. In other embodiments, the sequence encodes other than ADP and excludes a sequence encoding only ADP. As is well known in the art, the ADP coding region is located in the E3 region within the adenoviral genome from about 29468 bp to about 29773 bp; including the Y leader, the location of ADP is from about 28375 bp to about 29773 bp for Ad5. Other ADP regions for other serotypes are known in the art. An E3 sequence includes, but is not limited to, deletions; insertions; fusions; and substitutions. An E3 sequence may also comprise an E3 region or a portion of the E3 region. It is understood that, as an "E3 sequence" is not limited to an "E3 region", alternative references herein to an "E3 region" or "E3 sequence" do not indicate that

these terms are interchangeable. Assays for determining a functional E3 sequence for purposes of this invention are described herein.

The E4 gene has a number of transcription products and encodes two polypeptides (the products of open reading frames (ORFs) 3 and 6) which are responsible for stimulating the replication of viral genomic DNA and stimulating late gene expression, through interaction with heterodimers of cellular transcription factors E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55 kD protein for activity while the ORF 3 protein does not. In the absence of functional ORF 3- and ORF 6-encoded proteins, efficiency of plaque formation is less than 10^{-6} that of wild type virus.

To further increase cell-specificity of replication, it is possible to take advantage of the interaction between the E4 ORF 6 gene product and the E1B 55 kD protein. For example, if E4 ORFs 1-3 are deleted, viral DNA replication and late gene synthesis becomes dependent on E4 ORF6 protein. By generating such a deletion in a vector in which the E1B region is regulated by a cell-specific TRE, a virus is obtained in which both E1B and E4 functions are dependent on the cell-specific TRE which regulates E1B.

Late genes relevant to the disclosed vectors are L1, L2 and L3, which encode proteins of the virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. All late genes are under the control of the major late promoter (MLP), which is located in Ad5 between nucleotides 5986 and 6048.

In one embodiment, an adenovirus early gene is under transcriptional control of a cell specific, heterologous TRE. In additional embodiments, the early gene is selected from the group including E1A, E1B, E2, E3, E4. In another embodiment, an adenovirus late gene is under transcriptional control of a cell specific, heterologous TRE. In further embodiments, two or more early genes are under the control of heterologous TREs that function in the same target cell. The heterologous TREs can

be the same or different, or one can be a variant of the other. In additional embodiments, two or more late genes are under the control of heterologous TREs that function in the same target cell. The heterologous TREs can be the same or different, or one can be a variant of the other. In yet another embodiment, one or more early gene(s) and one or more late gene(s) are under transcriptional control of the same or different heterologous TREs, wherein the TREs function in the same target cell.

In some embodiments of the present invention, the adenovirus vector comprises the essential gene E1A and the E1A promoter is deleted. In other embodiments, the adenovirus vector comprises the essential gene E1A and the E1A enhancer I is deleted. In yet other embodiments, the E1A promoter is deleted and E1A enhancer I is deleted. In other embodiments, an internal ribosome entry site (IRES) is inserted upstream of E1B (so that E1B is translationally linked), and a target cell-specific TRE is operably linked to E1A. In still other embodiments, an (IRES) is inserted upstream of E1B (so that E1B is translationally linked), and target cell-specific TRE is operably linked to E1A, which may or may not maintain the E1A promoter and/or enhancer I (i.e., the E1A promoter and/or enhancer I may be, but not necessarily be, deleted). In yet other embodiments, the 19-kDa region of E1B is deleted.

For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and second gene are in frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used.

Adenovirus death protein (ADP) gene and gene product

In the construction of adenovirus vectors, the E3 region is often deleted to facilitate insertion of one or more TREs and/or transgenes. In some embodiments, however, the adenovirus death protein (ADP), encoded within the E3 region, is retained in an adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson *et al.* (1992) *J. Virol.* **66**:3633; and Tollefson *et al.* (1996) *J. Virol.* **70**:2296. Thus, inclusion of an ADP gene in a viral vector can render the vector more potent, making possible more effective treatment and/or a lower dosage requirement.

An ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which the ADP has been most fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and placed in operative linkage to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) is then introduced into an adenoviral genome, for example, in the E3 region, where expression of the ADP coding sequence will be driven by the MLP. The ADP coding sequence can, of course, also be inserted in other locations of the adenovirus genome, such as the E4 region. Alternatively, the ADP coding sequence can be operably linked to a heterologous TRE, including, but not limited to, another viral TRE or a target cell-specific TRE (see *infra*). In another embodiment, the ADP gene is present in a viral genome such that it is transcribed as part of a multi-cistronic mRNA in which its translation is associated with an IRES.

E3-containing target cell-specific adenoviral vectors

In some embodiments, the adenovirus vectors contain an E3 region, or a portion of an E3 region. Inclusion of the E3 region of adenovirus can enhance cytotoxicity of the target cell-specific adenoviral vectors of the present invention. Adenoviral vectors containing an E3 region may maintain their high level of

specificity and can be (a) significantly more cytotoxic; (b) produce higher virus yield including extracellular virus yield; (c) form larger plaques; (d) produce rapid cell death; and (e) kill tumors more efficiently *in vivo* than vectors lacking the E3 region. The adenoviral vectors of this invention may contain the E3 region or a portion of the E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example, increased replication and production, functionally equivalent (in which functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be constructed. For example, portions of E3 may be used. A portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gpl9k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex down regulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A ninth protein having a molecular weight of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in FIG. 6. These intact, portions, or variants of E3 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used.

In the adenovirus vectors of the present invention, E3 may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a target cell-specific TRE. Accordingly, in one embodiment, the invention provides an adenoviral vector, preferably replication competent, that comprises E3 region (or a portion of E3) under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a native adenoviral TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a target cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a target cell-specific TRE.

Transgenes under transcriptional control of a target cell-specific TRE

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having a single or multiple adenovirus gene(s) under control of a target cell-specific TRE, a transgene(s) is/are also under control of a target cell-specific TRE and optionally under translational control of an IRES. Transgenes include, but are not limited to, therapeutic transgenes and reporter genes.

Reporter genes

For example, a target cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and this construct may further comprise a second co-transcribed gene under translational control of an IRES. The second gene may be a reporter gene. The reporter gene can encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β-galactosidase (encoded by the *lacZ*

gene), luciferase, alkaline phosphatase, a green fluorescent protein, and horse radish peroxidase. For detection of a putative cancer cell(s) in a biological sample, the biological sample may be treated with modified adenoviruses in which a reporter gene (e.g., luciferase) is under control of a target cell-specific TRE. The target cell-specific TRE will be transcriptionally active in cells that allow the target cell-specific TRE to function, and luciferase will be produced. This production will allow detection of target cells, including cancer cells in, for example, a human host or a biological sample. Alternatively, an adenovirus can be constructed in which a gene encoding a product conditionally required for survival (e.g., an antibiotic resistance marker) is under transcriptional control of a target cell-specific TRE. When this adenovirus is introduced into a biological sample, the target cells will become antibiotic resistant. An antibiotic can then be introduced into the medium to kill the non-cancerous cells.

Therapeutic transgenes

Transgenes also include genes which may confer a therapeutic effect, such as enhancing cytotoxicity so as to eliminate unwanted target cells. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992)

Neuron 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. awsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN-α, -β, -χ, TNF-α, -β, TGF-α, -β, NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

Host cells

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Host systems are known in the art and need not be described in detail herein. Prokaryotic host cells include bacterial cells, for example, *E. coli, B. subtilis,* and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Examples of fungi (including yeast) host cells are *S. cerevisiae, Kluyveromyces lactis (K. lactis),* species of *Candida* including *C. albicans* and *C. glabrata, Aspergillus nidulans, Schizosaccharomyces pombe (S. pombe), Pichia pastoris*, and *Yarrowia lipolytica.* Examples of mammalian cells are cultured human target cells (HUC), KU-1, MYP3 (a non-tumorigenic rat target cell line), 804G (rat bladder carcinoma cell line), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. COS cells, mouse L cells,

LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used.

Compositions and kits

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Compositions can comprise an adenoviral vector(s) of the invention and a suitable solvent, such as a physiologically acceptable buffer. These are well known in the art. In other embodiments, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic or local administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 19th Edition, Mack Publishing (1995). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of bladder cancer cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a target cell-specific TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one or more early replication genes (although late gene(s) can be used). A target cell-specific TRE can be prepared using oligonucleotide synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a target cell-specific TRE. Accordingly, convenient restriction sites for annealing (i.e., inserting) a target cell-specific TRE can be engineered onto the 5' and 3' ends of a UP-TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, recombinant methods and/or obtained from biological sources.

Adenoviral vectors containing all replication-essential elements, with the desired elements (e.g., E1A) under control of a target cell-specific TRE, are conveniently prepared by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the other plasmid providing the right-hand region, one or more of which contains at least one adenovirus gene under control of a target cell-specific TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated,

followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a target cell-specific TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 (Bett et al. (1994); Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides room in the virus to insert a 3 kb target cell-specific TRE without deleting the endogenous enhancer/promoter. The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 provides an even larger E3 deletion (an additional 0.3 kb is deleted). Bett et al. (1994). Alternatively, the use of pBHGE3 (Microbix Biosystems, Inc.) provides the right hand end of Ad5, with a full-length of E3.

For manipulation of the early genes, the transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A coding segment is at 560 in the virus genome. This region can be used for insertion of a target cell-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a nucleotide sequence

change resulting in a unique restriction site, one can provide for insertion of target cell-specific TRE at that site.

A similar strategy may also be used for insertion of a target cell-specific TRE element to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a target cell-specific TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-specific response element regulating E1A, as the template for introducing a target cell-specific TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. In additional embodiments, the 19-kDa region of E1B is deleted.

Similarly, a target cell-specific TRE can be inserted upstream of the E2 gene to make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Immunol.* (1995) 199(part 3):177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a target cell-specific TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow target cell-restricted expression of E2 transcripts.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at about nt 35605, the TATA box at about nt 35631 and the first AUG/CUG of ORF I is at about nt 35532. Virtanen et al. (1984) *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a UP-TRE may be introduced upstream from the transcription start site. For the construction of full-length adenovirus with a target cell-specific TRE inserted in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc. Natl. Acad. Sci.* 80:5383–5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

Adenoviral constructs containing an E3 region can be generated wherein homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-containing adenoviral plasmid, is carried out.

Alternatively, an adenoviral vector comprising an E3 region can be introduced into cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus containing an E3 region. In this case, the E3-containing adenoviral vector and the adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

Alternatively, an E3-containing adenoviral vector of the invention can be constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard techniques of molecular biology.

Insertion of an IRES into a vector is accomplished by methods and techniques that are known in the art and described herein *supra*, including but not limited to, restriction enzyme digestion, ligation, and PCR. A DNA copy of an IRES can be

obtained by chemical synthesis, or by making a cDNA copy of, for example, a picornavirus IRES. *See*, for example, Duke et al. (1995) *J.Vvirol.* 66(3):1602-9) for a description of the EMCV IRES and Huez et al. (1998), *Mol. Cell. Biol.* 18(11):6178-90) for a description of the VEGF IRES. The internal translation initiation sequence is inserted into a vector genome at a site such that it lies upstream of a 5'-distal coding region in a multicistronic mRNA. For example, in a preferred embodiment of an adenovirus vector in which production of a bicistronic E1A-E1B mRNA is under the control of a target cell-specific TRE, the E1B promoter is deleted or inactivated, and an IRES sequence is placed between E1A and E1B. IRES sequences of cardioviruses and certain aphthoviruses contain an AUG codon at the 3' end of the IRES that serves as both a ribosome entry site and as a translation initiation site. Accordingly, this type of IRES is introduced into a vector so as to replace the translation initiation codon of the protein whose translation it regulates. However, in an IRES of the entero/rhinovirus class, the AUG at the 3' end of the IRES is used for ribosome entry only, and translation is initiated at the next downstream AUG codon. Accordingly, if an entero/rhinovirus IRES is used in a vector for translational regulation of a downstream coding region, the AUG (or other translation initiation codon) of the downstream gene is retained in the vector construct.

Methods of packaging polynucleotides into adenovirus particles are known in the art and are also described in co-owned PCT PCT/US98/04080.

Delivery of Adenovirus vectors

The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs. Adenoviral vectors can, alternatively, comprise polynucleotide constructs that are complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or

dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

If an adenoviral vector comprising an adenovirus polynucleotide is packaged into a whole adenovirus (including the capsid), the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virol.* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus(or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14}. The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μg to about 1000 μg of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard

knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

Methods using the adenovirus vectors of the invention

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

In one embodiment, methods are provided for conferring selective cytotoxicity in cells that allow a target cell-specific TRE to function, preferably target cells, comprising contacting such cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ^{3}H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow a target cell-specific TRE to function, preferably target cells, preferably cancer cells. These methods entail combining an adenovirus vector with the cells, whereby said adenovirus is propagated.

Another embodiment provides methods for killing cells that allow a target cell-specific TRE to function in a mixture of cells, comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells that allow a target cell-specific TRE to function, and can be an *in vivo* mixture or *in vitro* mixture.

The invention also includes methods for detecting cells which allow a target cell-specific TRE to function, such as cancer cells, in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively, the sample can be contacted with an adenovirus in which a reporter gene is under control of a target cell-specific TRE. When such an adenovirus is introduced into a biological

sample, expression of the reporter gene indicates the presence of cells that allow a target cell-specific TRE to function. Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under control of a target cell-specific TRE. This gene may encode, for example, antibiotic resistance. Later the biological sample is treated with an antibiotic. The presence of surviving cells expressing antibiotic resistance indicates the presence of cells capable of target cell-specific TRE function. A suitable biological sample is one in which cells that allow a target cell-specific TRE to function, such as cancer cells, may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells that allow a target cell-specific TRE to function, such as carcinoma cells, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, cells that allow a target cell-specific TRE to function can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, preferably a tumor cell that allows a target cell-specific TRE to function, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. For these methods, the adenoviral vector may or may not be used in

conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells that allow a target cell-specific TRE to function. Tumor cell markers include, but are not limited to, CK-20. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with cancer as described above. Also indicated are individuals who are considered to be at risk for developing cancer (including single cells), such as those who have had disease which has been resected and those who have had a family history of cancer. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for

tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The amount of adenoviral vector(s) to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular target cell-specific TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under target cell-specific TRE control) as well as whether the adenoviral vector is used in conjunction with other treatment modalities.

If administered as a packaged adenovirus, from about 10^4 to about 10^{14}, preferably from about 10^4 to about 10^{12}, more preferably from about 10^4 to about 10^{10}. If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01 μg to about 100 μg can be administered, preferably 0.1 μg to about 500 μg, more preferably about 0.5 μg to about 200 μg. More than one adenoviral vector can be administered, either simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective. Examples of chemotherapeutics which are suitable for suppressing bladder tumor growth are BGC (bacillus Calmett-Guerin); mitomycin-C; cisplatin; thiotepa; doxorubicin; methotrexate; paclitaxel (TAXOL™); ifosfamide; gallium nitrate; gemcitabine; carboplatin; cyclosphasphamid; vinblastine; vincristin; fluorouracil; etoposide; bleomycin. Examples of combination therapies include (CISCA (cyclophosphamide, doxorubicin, and cisplatin); CMV (cisplatin, methotrexate, vinblastine); MVMJ (methodtrextate, vinblastine, mitoxantrone, carboplain); CAP (cyclophosphamide, doxorubicin, cisplatin); MVAC (methotrexate, vinblastine, doxorubicin, cisplatin). Radiation may also be combined with chemotherapeutic agent(s), for example, radiation with cisplatin. Administration of the

chemotherapeutic agents is generally intravesical (directly into the bladder) or intravenous.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

Example 1: Construction of a replication-competent adenovirus vector comprising an AFP-TRE and an EMCV IRES

The encephalomyocarditis virus (ECMV) IRES as depicted in Table 1 was introduced between the E1A and E1B regions of a replication-competent adenovirus vector specific for cells expressing AFP as follows. Table 1 shows the 519 base pair IRES segment which was PCR amplified from Novagen's pCITE vector by primers A/B as listed in Table 4. A 98 base pair deletion in the E1A promoter region was created in PXC.1, a plasmid which contains the left-most 16 mu of Ad5. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5, from Adenovirus 5 nt 22 to 5790 including the inverted terminal repeat, the packaging sequence, and the E1a and E1b genes in vector pBR322. pBHG10 (Bett. et al. (1994) Proc. Natl. Acad. Sci. USA 91:8802-8806; Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The resultant plasmid, CP306 (PCT/US98/16312), was used as the backbone in overlap PCR to generate CP624. To place a SalI site between E1a and E1b, primers C/D, E/F (Table 4) were used to amplify CP306, plasmid derived from pXC.1 and lacking the E1a promoter. After first round PCR using CP306 as template and primers C/D, E/F, the resultant two DNA fragments were mixed together for another round of overlapping PCR with primers C/F. The overlap PCR product was cloned by blunt end ligation to vector. The resultant plasmid, CP624 (Table 5), contains 100 bp deletion in E1a/E1b intergenic region and introduces SalI site into the junction. On this plasmid, the endogenous E1a promoter is deleted, and the E1a polyadenylation signal and the E1b promoter are replaced by the SalI site. Next, the SalI fragment of CP625 was cloned

into the SalIsite in CP624 to generate CP627 (Table 5). CP627 has an EMCV IRES connecting adenovirus essential genes E1a and E1b. In CP627, a series of different tumor-specific promoters can be placed at the *PinA*I site in front of E1a to achieve transcriptional control on E1 expression.

Table 4

Primer	Sequence	Note
A.	5'-GACGTCGACTAATTCCGGTTATTTTCCA	For PCR EMCV IRES, *GTCGAC* is a SalI site.
B.	5'-GACGTCGACATCGTGTTTTTCAAAGGAA	For PCR EMCV IRES, *GTCGAC* is a SalI site.
C.	5'-CCTGAGACGCCCGACATCACCTGTG	Ad5 sequence to 1314 to 1338.
D.	5'-GTCGACCATTCAGCAAACAAAGGCGTTAAC	Antisense of Ad5 sequence 1572 to 1586. *GTCGAC* SalI site. Underline region overlaps with E.
E.	5'-TGCTGAATGGTCGACATGGAGGCTTGGGAG	Ad5 sequence 1714 to 1728. *GTCGAC* is a SalI site. Underline region overlaps with D.
F.	5'-CACAAACCGCTCTCCACAGATGCATG	Antisense of Ad5 sequence 2070 to 2094.

For generating a liver cancer-specific virus, an about 0.8kb AFP promoter fragment as shown in Table 3 was placed into the *Pin*AI site of CP627 thereby yielding plasmid CP686. Full-length viral genomes were obtained by recombination between CP686 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus recombination were pBHGE3 (Microbix Biosystems Inc.), containing an intact E3 region, and pBHG11 or pBHG10 (Bett et al. (1994) containing a deletion in the E3 region.

The virus obtained by recombination of CP686 with a right arm containing an intact E3 region was named CV890. The virus obtained by recombination of CP686 with a right arm containing a deleted E3 region (pBHG 10) was named CV840. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus vector designated CV890 comprises 0.8 kb AFP promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion of the E1B promoter and an intact E3 region. Adenovirus vector CV840 comprises AFP

promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B promoter and a deleted E3 region.

Table 5

Plasmid designation	Brief description
CP306	An E1A promoter deleted plasmid derived from pXC.1
CP624	Overlap PCR product from CP306 to generate 100 bp deletion and introduce a SalI site at E1A and E1B junction; E1A and E1B promoter deleted in E1A/E1B intergenic region.
CP625	EMCV IRES element ligated to PCR-blunt vector (Invitrogen pCR® blunt vector).
CP627	IRES element derived from CP625 by *Sal*I digestion and ligated to CP624 SalI site placing IRES upstream from E1B.
CP628	Probasin promoter derived from CP251 by *PinA*I digestion and cloned into *PinA*I site on CP627.
CP629	HCMV IE promoter amplified from pCMV beta (Clontech) with *PinA*I at 5' and 3' ends ligated into CP627 *PinA*I site.
CP630	A 163 bp long VEGF IRES fragment (Table 1) cloned into the *Sal*I site on CP628.
CP686	AFP promoter from CP219 digested with *PinA*I and cloned into *PinA*I site on CP627.

Example 2: Construction of a replication-competent adenovirus vector with a probasin TRE and an EMCV IRES

The probasin promoter as shown in Table 3 was inserted at the PinAI site of plasmid CP627 (see Example 1) to generate CP628, which contains a probasin promoter upstream of E1A and an EMCV IRES between E1A and E1B. Full-length viral genomes were obtained by recombination between CP628 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus recombination were pBHGE3, containing an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus designated CV 834 comprises probasin promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B, a deletion of the E1B endogenous promoter and a deleted E3 region.

Example 3: Construction of a replication-competent adenovirus vector with a hCMV-TRE and an EMCV IRES

The hCMV immediate early gene (IE) promoter from plasmid CP629, originally derived from pCMVBeta (Clonetech, Palo Alto) was inserted at the PinAI site of plasmid CP627 (see Example 1) to generate CP629, containing a CMV IE promoter upstream of E1A and an IRES between E1A and E1B. Full-length viral genomes were obtained by recombination between CP629 and a plasmid containing a right arm of an adenovirus genome. The right arms used in virus recombination were pBHGE3, containing an intact E3 region, and pBHG11 or pBHG10 containing a deletion in the E3 region. The structure of all viral genomes was confirmed by conducting PCR amplifications that were diagnostic for the corresponding specific regions.

Therefore, adenovirus vector designated CV835 comprises hCMV-IE promoter, E1A, a deletion of the E1A promoter, EMCV IRES, E1B a deletion in the E1B endogenous promoter and a deleted E3 region. CV835 lacks the hCMV enhancer and is therefore not tissue specific. By adding the hCMV IE enhancer sequence to CV835, the vector is made tissue specific.

Example 4: Replication of IRES-containing adenovirus vectors with different TREs controlling E1 expression

The viral replication of adenovirus vectors comprising the probasin promoter (CV836 and CV834) generally considered a weak promoter, and the human cytomegalovirus immediate early gene (HCMV-IE) promoter (CV837 and CV835), generally considered a strong promoter, were characterized in the virus yield assay.

Probasin promoter containing adenovirus vectors (see PCT/US98/04132), CV836 and CV834, and HCMV-IE promoter containing adenovirus vectors, CV837 and CV835, were tested against a panel of cell lines for viral replication (indicative of lethality) and specificity. Cell lines 293 (the producer line), LNCap and HepG2 were plated at 0.5 x 10^6 per well in 6 well tissue culture plates, incubated for 24 hours at 37° C, then infected with CV836, CV834 or CV837 and CV835 at a multiplicity of infection (MOI) of 2 plaque forming units per cell (PFU/cell) for 4 hours at 37° C. At the end of the infection period, the medium was replaced and the cells were incubated at 37° C for a further 72 hours before harvesting for a viral yield assay as described in Yu et al. (1999) *Cancer Res.* 59:1498-1504. The results are shown in Fig. 3.

The data demonstrate that the presence of an IRES element in the intergenic region between E1A and E1B does not significantly affect viral replication, as compared to control viruses lacking an IRES, such as a wild-type AD5 with a deletion in the E3 region. In CV834, the loss of tissue cytotoxicity could be caused by the weakness of the probasin promoter in the virus structure.

Example 5: Comparison of dual TRE vectors with single TRE/IRES-containing vectors

Two liver cancer-specific adenovirus vectors, CV790 and CV733 (also designated CN790 and CN733, respectively), were generated and characterized. See PCT/US98/04084. These viruses contain two AFP TREs, one upstream of E1A and one upstream of E1B. They differ in that CV790 contains an intact E3 region, while the E3 region is deleted in CV733. Replication of these two viruses was compared with that of the newly generated IRES-containing viruses, CV890 and CV840 (*see* Example 1).

Virus replication was compared, in different cell types, using a virus yield assay as described in Example 4. Cells were infected with each type of virus and,

72 hrs after infection, virus yield was determined by a plaque assay. Figs. 4A and 4B show viral yield for different viruses in different cell types. The results indicate that vectors containing an IRES between E1A and E1B (CV890 and CV840), in which E1B translation is regulated by the IRES, replicate to similar extents as normal adenovirus and viruses with dual AFP TREs, in AFP-producing cells such as 293 cells and hepatoma cells. In SK-Hep-1 (liver cells), PA-1 (ovarian carcinoma) and LNCaP cells (prostate cells) the IRES-containing viruses do not replicate as well as dual TRE or wild-type adenoviruses, indicating that the IRES-containing viruses have higher specificity for hepatoma cells. Based on these results, it is concluded that IRES-containing vectors have unaltered replication levels, but are more stable and have better target cell specificity, compared to dual-TRE vectors.

Example 6: Uroplakin adenoviral constructs containing an EMCV IRES

A number of E3-containing viral constructs were prepared which contained uroplakin II sequences (mouse and/or human) as well as an EMCV internal ribosome entry site (IRES). The viral constructs are summarized in Table 6. All of these vectors lacked an E1A promoter and retained the E1A enhancer.

The 519 base pair EMCV IRES segment was PCR amplified from Novagen's pCITE vector by primers A/B:

primer A: 5'-GACGTCGACTAATTCCGGTTATTTTCCA

primer B 5'-GACGTCGACATCGTGTTTTTCAAAGGAA (*GTCGAC* is a SalI site).

The EMCV IRES element was ligated to PCR blunt vector (Invitrogen pCR® blunt vector).

CP1066

The 1.9kb-(-1885 to +1) fragment of mouse UPII from CP620 was digested with AflIII (blunted) and HindIII and inserted into pGL3-Basic from CP620 which had been digested with XhoI (blunted) and HindIII to generate CP1066.

CP1086

The 1.9kb mouse UPII insert was digested with PinAI and ligated with CP269 (CMV driving E1A and IRES driving E1B with the deletions of E1A/E1B endogenous promoter) which was similarly cut by PinAI.

CP1087

The 1kb (-1128 to +1) human UPII was digested with PinAI from CP665 and inserted into CP629 which had been cut by PinAI and purified (to elute CMV).

CP1088

The 2.2kb (-2225 to +1) human UPII was amplified from CP657 with primer 127.2.1 (5'-AGGACCGGTCACTATAGGGCACGCGTGGT-3') PLUS 127.2.2 (5'-AGGACCGGTGGGATGCTGGGCTGGGAGGTGG-3') and digested with PinAI and ligated with CP629 cut with PinAI.

CP627 is an Ad5 plasmid with an internal ribosome entry site (IRES) from encephelomycarditis virus (EMCV) at the junction of E1A and E1B. First, CP306 (Yu et al., 1999) was amplified with primer pairs 96.74.3/96.74.6 and 96.74.4/96.74.5.

The two PCR products were mixed and amplified with primer pairs 96.74.3 and 96.74.5. The resultant PCR product contains a 100bp deletion in E1A-E1B intergenic region and a new SalI site at the junction. EMCV IRES fragment was

amplified from pCITE-3a(+) (Novagen) using primers 96.74.1 and 96.74.2. The SalI fragment containing IRES was placed into SalI site to generate CP627 with the bicistronic E1A-IRES-E1B cassette. CP629 is a plasmid with CMV promoter amplified from pCMVbeta (Clontech) with primer 99.120.1 and 99.120.2 and cloned into PinAI site of CP627.

CP657 is a plasmid with 2.2kb 5' flanking region of human UP II gene in pGL3-Basic (Promega). The 2.2kb hUPII was amplified by PCR from GenomeWalker product with primer 100.113.1 and 100.113.2 and TA-cloned into pGEM-T to generate CP655.

The 2.2kb insert digested from SacII (blunt-ended) and KpnI was cloned into pGL3-Basic at HindIII (blunted) and KpnI to create CP657.

CP1089

The 1kb (-965 to +1) mouse UPII was digested by PinAI from CP263 and inserted into CN422 (PSE driving E1A and GKE driving E1B with the deletions of E1A/E1B endogenous promoter) cut by PinAI and purified and further digested with EagI and ligated with 1kb (-1128 to +1) human UPII cut from CP669 with EagI.

CP1129

The 1.8kb hUPII fragment with PinAI site was amplified from CP657 with primer 127.50.1 and 127.2.2 and cloned into PinAI site of CP629.

CP1131

CP686 was constructed by replacing the CMV promoter in CP629 with an AFP fragment from CP219. A 1.4kb DNA fragment was released from CP686 by digesting it with BssHII, filling with Klenow, then digesting with BglII. This DNA fragment was then cloned into a similarly cut CP686 to generate CP1199. In CP1199, most of the E1B 19-KDa region was deleted. The 1.8kb hUPII fragment

with PinAI site was amplified from CP657 by PCR with primer 127.50.1 and 127.2.2 and inserted into similarly digested CP1199 to create CP1131.

The plasmids above were all co-transfected with pBHGE3 to generate CV874 (from CP1086), CV875 (from CP1087), CV876 (from 1088) and CV877 (from CP1089), CV882 (from CP1129) and CV884 (from CP1131). CP1088, CP1129 and CP1131 were cotransfected with pBHGE3 for construction of CV876, CV892 and CV884, respectively by lipofectAMINE (Gibco/BRL) for 11-14 days. pBHGE3 was purchased from Microbix, Inc., and was described previously. The cells were lysed by three freeze-thaw cycles and plaqued on 293 cells for a week. The single plaques were picked and amplified by infection in 293 cells for 3-5 days. The viral DNAs were isolated from the lysates and the constructs were confirmed by PCR with primer 31.166.1/ 51.176 for CV876 and primer 127.50.1/51.176 for CV882 and CV884 at E1 region and primer 32.32.1/2 for all three viruses at E3 region.

TABLE 6

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV874	CP1086	pBHGE3	1.9 kb mUPII	IRES	intact
CV875	CP1087	pBHGE3	1.0 kb hUPII	IRES	intact
CV876	CP1088	pBHGE3	2.2 kb hUPII	IRES	intact
CV877	CP1089	pBHGE3	1.0 kb mUPII	1.0 kb hUPII (E1B promoter deleted)	intact
CV882	CP1129	pBHGE3	1.8 kb hUPII	IRES	intact
CV884	CP1131	pBHGE3	1.8 kb hUPii	IRES (E1B 19-kDa deleted)	intact

Viruses are tested and characterized as described above.

Primer sequences:

96.74.1	GACGTCGACATCGTGTTTTTCAAAGGAA
96.74.2	GACGTCGACTAATTCCGGTTATTTTCCA
96.74.3	CCTGAGACGCCCGACATCACCTGTG
96.74.4	TGCTGAATGGTCGACATGGAGGCTTGGGAG
96.74.5	CACAACCGCTCTCCACAGATGCATG
96.74.6	GTCGACCATTCAGCAAACAAAGGCGTTAAC
100.113.1	AGGGGTACCCACTATAGGGCACGCGTGGT
100.113.2	ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG
127.2.2	AGGACCGGTGGGATGCTGGGCTGGGAGGTGG
127.50.1	AGGACCGGTCAGGCTTCACCCCAGACCCAC
31.166.1	TGCGCCGGTGTACACAGGAAGTGA
32.32.1	GAGTTTGTGCCATCGGTCTAC
32.32.2	AATCAATCCTTAGTCCTCCTG
51.176	GCAGAAAAATCTTCCAAACACTCCC
99.120.1	ACGTACACCGGTCGTTACATAACTTAC
99.120.2	CTAGCAACCGGTCGGTTCACTAAACG

Example 7: Construction of a replication-competent adenovirus vector with a tyrosinase TRE and EMCV IRES

CP621 is a plasmid containing a human tyrosinase enhancer and promoter elements in a PinAl fragment. This fragment is ligated to the PinAl site on CP627 to generate CP1078. CP1078 is combined with pBHGE3 to generate a new melanoma specific virus, CV859. Table 3 depicts the polynucleotide sequence of the PinAl fragment which contains a tyrosinase promoter and enhancer.

Example 8: Construction of a replication-competent adenovirus vector with a probasin-TRE and a VEGF IRES

Using a strategy similar to that described in Example 1, the IRES fragment from the mouse vascular endothelial growth factor (VEGF) gene is amplified and cloned into CP628 at the SalI site. Table 1 depicts the IRES fragment obtainable from vascular endothelial growth factor (VEGF) mRNA. In order to clone this

fragment into the E1a/E1b intergenic region, two pieces of long oligonucleotide are synthesized. The sense oligonucleotide is shown in the Table, whereas the second piece is the corresponding antisense one. After annealing the two together to create a duplex, the duplex is subjected to SalI digestion and the resulting fragment is cloned into the SalI site on CP628. The resulting plasmid, CP630, has a probasin promoter in front of E1a and an VEGF IRES element in front of E1b. This plasmid is used to construct a prostate cancer-specific virus comprising the VEGF IRES element.

Example 9: Construction of a replication-competent adenovirus vector with an AFP-TRE and a VEGF IRES

Using a strategy similar to Example 1, a PinAI fragment which contains AFP TRE can be obtained. This AFP TRE is cloned into the PinA1 site in front of E1A on CP628 yielding plasmid CP1077. This plasmid has the AFP TRE for E1 transcriptional control and the VEGF IRES element before E1b. CP1077 can be recombined with pBHGE3 to generate a liver-specific adenovirus, designated as CV858.

Example 10: Construction of a replication-competent adenovirus vector with a hKLK2-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from human glandular kallikrein II as shown in Table 3 was cloned into the PinAI site in CP627. The resultant plasmid, CP1079, is cotransfected with pBHGE3 to create CV860.

Example 11: Treatment of Hep3B Tumor Xenografts with Replication-Competent Hepatoma Specific CV790 and Doxorubicin and Hepatoma Specific CV890 and Doxorubicin

CV790 is an AFP producing hepatocellular carcinoma specific adenovirus, with EIA and E1B under the control of an identical AFP promoter and enhancer (822

base pair promoter shown in Table 3) with an E3 region. The CV890 adenovirus construct is also a hepatoma or liver-specific adenoviral mutant with the E1A and E1B genes under transcriptional control of 822bp AFP promoter (827 bp including nucleotides for restriction site), wherein E1B is under translational control of EMCV IRES and having an intact E3 region. The structure of CV890 therefore reads as AFP/E1A, IRES/E1B, E2, E3, E4. *In vivo* studies of the efficacy of combinations of CV790 and doxorubicin and CV890 and doxorubicin were performed according to the protocols described in detail in Example 4, with minor alterations which are described below.

Xenografts in the study of CV790 and CV890 combined with chemotherapeutic agents utilized liver carcinoma Hep3B cells. Virus, CV790 or CV890, was administered by a single intravenous injection of 1×10^{11} particles through the tail veins of the nude mice. One day after virus delivery, a single dose of doxorubicin was given to each animal by i.p. injection. The doxorubicin dose was 10 mg/kg for both doxorubicin alone and doxorubicin combined with virus treatments. Tumor volume was measured once a week for six weeks. Tumors were measured weekly in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width $(mm)^2$]/2.

Figure 8 depicts the anti-tumor activity of CV890 containing an IRES as compared to CV 790 containing dual TREs. As Figure 8 demonstrates, relative tumor volume was less with administration of CV890 than administration of CV790.

Furthermore, both CV790/doxorubicin and CV890/doxorubicin treatment of the hepatoma showed synergistic results. Four days after treatment with either CV790/doxorubicin or CV890/doxorubicin the relative tumor volume was less than 10%. Unlike mice treated with either virus alone or doxorubicin alone, after day 4, the relative tumor volume did not increase for either the either CV790/doxorubicin or CV890/doxorubicin treated mice. At day 6 in the control mice, the relative tumor volume was approximately 1000% in the CV790 study and approximately 600% in

the CV890 study. The relative tumor volumes of mice treated with virus alone were 250% (CV790) and 520% (CV890) while the relative tumor volumes for mice treated with doxorubicin alone were 450% with 280% in the CV790 study and 500% in the CV890 study.

Example 12: *In vitro* characterization of melanocyte-specific TRE-containing adenoviral constructs

An especially useful objective in the development of melanocyte cell-specific adenoviral vectors is to treat patients with melanoma. Methods are described below for measuring the activity of a melanocyte-specific TRE and thus for determining whether a given cell allows a melanocyte-specific TRE to function.

Cells and Culture Methods

Host cells such as, HepG2 (liver); Lovo (colon); LNCaP (prostate); PMEL (melanoma); SKMel (melanoma); G361 (melanoma) and MeWo cells are obtained at passage 9 from the American Type Culture Collection (Rockville, MD). MeWo cells are maintained in RPMI 1640 medium (RPMI) supplemented with 10% fetal bovine serum (FBS; Intergen Corp.), 100 units/mL of penicillin, and 100 units/mL streptomycin. MeWo cells being assayed for luciferase expression are maintained in 10% strip-serum (charcoal/dextran treated fetal bovine serum to remove T3, T4, and steroids; Gemini Bioproduct, Inc., Calabasas, CA) RPMI.

Transfections of MeWo Cells

For transfections, MeWo cells are plated out at a cell density of 5×10^5 cells per 6-cm culture dish (Falcon, NJ) in complete RPMI. DNAs are introduced into MeWo cells after being complexed with a 1:1 molar lipid mixture of N-[1-(2,3-dioleyloxy)propyl-*N,N,N*-trimethylammonium chloride (DOTAP™; Avanti Polar Lipids, AL) and dioleoyl-phosphatidylethanolamine (DOPE™; Avanti Polar Lipids, AL); DNA/lipid complexes are prepared in serum-free RPMI at a 2:1 molar ratio. Typically, 8 μg (24.2 nmole) of DNA is diluted into 200 μL of incomplete RPMI and

added dropwise to 50 nmole of transfecting, lipids in 200 μL of RPMI with gentle vortexing to insure homogenous mixing of components. The DNA/lipid complexes are allowed to anneal at room temperature for 15 minutes prior to their addition to MeWo cells. Medium is removed from MeWo cells and replaced with 1 mL of serum-free RPMI followed by the dropwise addition of DNA/lipid complexes. Cells are incubated with complexes for 4-5 hours at 37°C, 5% CO_2. Medium was removed and cells washed once with PBS. The cells were then trypsinized and resuspended in 10% strip-serum RPMI (phenol red free). Cells were replated into an opaque 96-well tissue culture plate (Falcon, NJ) at a cell density of 40,000 cells/well per 100 μL media and assayed.

Plaque assays

To determine whether the adenoviral constructs described above replicate preferentially in melanocytes, plaque assays are performed. Plaquing efficiency is evaluated in the following cell types: melanoma cells (MeWo), prostate tumor cell lines (LNCaP), breast normal cell line (HBL-100), ovarian tumor cell line (OVCAR-3, SK-OV-3), and human embryonic kidney cells (293). 293 cells serve as a positive control for plaquing efficiency, since this cell line expresses Ad5 ElA and ElB proteins. For analyzing constructs comprising a melanocyte-specific TRE, cells that allow a melanocyte-specific TRE to function, such as the cell lines provided above and cells that do not allow such function, such as HuH7, HeLa, PA-1, or G361, are used. The plaque assay is performed as follows: Confluent cell monolayers are seeded in 6-well dishes eighteen hours before infection. The monolayers are infected with 10-fold serial dilutions of each virus. After infecting monolayers for four hours in serum-free media (MEM), the medium is removed and replaced with a solution of 0.75% low melting point agarose and tissue culture media. Plaques are scored two weeks after infection.

Example 13: Construction of a replication-competent adenovirus vector with a CEA-TRE and a EMCV IRES

Using a strategy similar to Example 1, the TRE fragment from Carcinembryonic antigen (CEA)(Table 3, SEQ ID NO:14) is used to construct virus designated CV873. A PinAI fragment containing the CEA-TRE was cloned into the PinAI site in front of E1A CP627 for the transcriptional control. The resultant plasmid CP1080 is used together with pBHGE3 to generate CV873.

Example 14: *In vitro* and *In vivo* assays of anti-tumor activity

An especially useful objective in the development of urothelial cell-specific adenoviral vectors is to treat patients with bladder cancer. An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against cell lines and tumor xenografts grown subcutaneously in Balb/c nu/nu mice.

***In vitro* characterization of CV876**

Virus yield assay for CV876

5 X $10^5$293, RT-4, SW780, PA-1, G361, MKNI, HBL-100, Fibroblast (from lung) and Smooth muscle cells (from bladder) were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Unlike wt. Ad5, CV802 which grows well in all of the cells tested, CV876 replicates much better in permissive cells (293, RT-4 and SW780) than in non-permissive cells (PA-1, G361, MKN1, HBL-100 and primary cells) by about 100-

10000 fold. Noticeably, the replication in SW780 for CV876 is about 100 fold less than CV802, which indicates the limitation of this virus in efficacy.

Growth curve experiment for CV876

5 X 10^5 RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with 133) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV876 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. However, CV876 does show a delay of replication in RT-4 compared to CV802.

Cytopathic effect assay for CV876

5 X $10^5$293, RT-4, SW780, PA-1, MKN1 and LNCap were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV876 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (PA-1, MKN-1 and LNCap).

MTT assay for CV876

2 X $10^4$293, RT-4, SW780, MKN1, PA-1, HBL-100, Smooth muscle cells (from bladder) and Fibroblast (from lung) were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV876 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time point of day 1, 3,5,7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV876 shows efficacy only in permissive cells but not in non-permissive cells. Again, in RT-4 and SW780, CV876 kills the cells much slower than CV802.

In vitro characterization of CV882

Virus yield assay for CV882

5 X $10^5$293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to CV802 (the difference is less than 100 fold) while it shows over 1000-

1000000 fold difference in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

Growth curve experiment for CV882

5 X 10^5RT-4, PA-1, and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three, freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV882 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. Additionally, CV882 shows better replication in RT-4 compared to CV876.

Cytopathic effect assay for CV882

5 X $10^5$293, RT-4, SW780, HBL-l 00, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPNI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV882 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (HBL-100, G361, PA-1 and Fibroblast cells).

MTT assay for CV882

2 X 10^4RT-4, SW780, PA-1, HBL-100, U118 and Fibroblast were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV882 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at lmg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for lh and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV882 shows efficacy only in permissive cells but not in non-permissive cells.

In Vitro Characterization of CV884

Virus yield assay for CV884

5 X $10^5$293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV984 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV884 is very similar as CV802 in permissive cells (293, RT-4 and SW780) but shows over 1000 fold difference with CV802 in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells). CV884 shows better efficacy than CV876 and CV882 without losing much specificity.

Growth curve experiment for CV884

5 X 10^5RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV884 replicates very well only in RT-4 (similar as CV802) but not in primary cells and PA-1. Again, the replication of CV884 is better than CV882 and CV876.

Cytopathic effect assay for CV884

5 X $10^5$293, RT-4, SW780, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV884 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (G361, PA-I and Fibroblast cells).

MTT assay for CV884

2 X $10^4$293, RT-4, SW780, U118, Fibroblast and Smooth muscle cells were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV884 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at lmg/ml solution which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for lh and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV884 shows strong efficacy (similar as wt. Ad5) only in permissive cells but not in non-permissive cells.

In vivo activity of CV808

Mice were given subcutaneous (SC) injections of 1 x 10^6 sW780 cells. When tumors grew to about 500 mm^3, CV808 was introduced into the mice (5 X 10^7 PFU of virus in 0.1 ml PBS and 10% glycerol) intratumorally. Control mice received vehicle alone. Tumor sizes were measured weekly. The results are shown in FIG. 11. The data indicate that CV808 was effective at suppressing tumor growth.

While it is highly possible that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of adenovirus vector can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of mice bearing bladder epithelial tumors are inoculated with 10^8 to 10^{10} PFU of an adenoviral vector by tail vein injection, or with buffer used to carry the virus as a negative control. The effect of IV injection of the adenoviral vector on tumor size is compared to vehicle treatment.

Example 15: Synergistic Effect of CV 890 with Chemotherapeutics

Materials and Methods

Cells. Hepatocellular carcinoma cell lines HepG2, Hep3B, PLC/PRF/5, SNU449, and Sk-Hep-1, Chang liver cell (human normal liver cells), as well as other tumor cell lines PA-1 (ovarian carcinoma), UM-UC-3 (bladder carcinoma), SW 780 (bladder carcinoma), HBL100 (breast epithelia), Colo 201 (Colon adenocarcinoma), U 118 MG (glioblastoma) and LNCaP (prostate carcinoma) were obtained from the American Type Culture Collection. HuH-7 (liver carcinoma) was a generous gift of Dr. Patricia Marion (Stanford University). 293 cells (human embryonic kidney containing the E1 region of Adenovirus) were purchased from Microbix, Inc. (Toronto, Canada). The primary cells nBdSMC (normal human bladder smooth muscle cells), nHLFC (normal human lung fibroblast cells), and nHMEC (normal human mammary epithelial cells) were purchased from Clonetics (San Diego, California). All tumor cell lines were maintained in RPMI 1640 (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (Irvine Scientific), 100 U/ml penicillin and 100 ug/ml streptomycin. Primary cells were maintained in accordance with vendor instructions (Clonetics, San Diego). Cells were tested for the expression of AFP by immunoassay (Genzyme Diagnostics, San Carlos, CA).

Virus yield and one-step growth curves. Six well dishes (Falcon) were seeded with $5x10^5$ cells per well of calls of interest 24 hrs prior to infection. Cells were infected at an multiplicity of infection (MOI) of 2 PFU/cell for three hours in serum-free media. After 3 hours, the virus containing media was removed, monolayers were washed three times with PBS, and 4 ml of complete media (RPMI1640 + 10% FBS) was added to each well. 72 hours post infection, cells were scraped into the culture medium and lysed by three cycles of freeze-thaw.

The one-step growth curves time points were harvested at various time points after infection. Two independent infections of each virus cell-combination were titered in duplicate on 293 cells (Yu et al., 1999, *Cancer Research*, 59:1498-1504.

Northern blot analysis. Hep3B or HBL100 cells were infected at an MOI of 20 PFU/cell (plaque forming unit per cell) with either CV802 or CV890 and harvested 24 hours post infection. Total cell RNA was purified using the RNeasy protocol (Qiagen). The Northern blot was conducted using NorthernMax Plus reagents (Ambion, Austin, Texas). 5ug of RNA was fractionated on a 1% agarose, formaldehyde-based denaturing gel and transferred to a BrightStar-Plus (Ambion) positively charged membrane by capillary transfer. The antisense RNA probes for E1A (adenovirus genome 501bp to 1141bp) or E1B (1540bp-3910bp) were PCR products cloned in pGEM-T easy (Promega) and transcription labeled with [α^{32}P] UTP. Blots were hybridized at 68°C for 14 hours with ZipHyb solution and washed using standard methods (Ambion). Membranes were exposed to BioMax film (Kodak).

Western blot analysis. Hep3B or HBL100 cells were infected at MOI of 20 PFU/cell with either CV802 or CV890 and harvested 24 hours post infection. Cells were washed with cold PBS and lysed for 30 min on ice in (50mM Tris, pH8.0, 150 mM NaCl, 1% IGEPAL CA360 a NP40 equivalent (Sigma), 0.5% sodium deoxycholate, and protease inhibitor cocktail from (Roche, Palo Alto, California). After 30 min centrifugation at 4C, the supernatant was harvested and the protein concentration determined with protein assay ESL kit (Roche). Fifty micrograms of protein per lane were separated on 816% SDS-PAGE and electroblotted onto Hybond ECL membrane (Amersham Pharmacia, Piscataway, New Jersey). The membrane was blocked overnight in PBST (PBS with 0.1% Tween-20) supplemented with 5% nonfat dry milk. Primary antibody incubation was done at room temperature for 2-3 hrs with PBST/1 % milk diluted antibody, followed by wash and 1 hr incubation with diluted horseradish peroxidase-conjugated secondary antibody (Santa Cruz

Biotechnology Inc., Santa Cruz, California). Enhanced chemiluminescence (ECL; Amersham Pharmacia) was used for the detection. E1A antibody (clone M58) was from NeoMarkers (Fremont, California), E1B-21 kD antibody was from Oncogene (Cambridge, Massachusetts). All antibodies were used according manufacturer's instruction.

Cell viability assay and statistical analysis. To determine the cell killing effect of virus and chemotherapeutic agent in combination treatment, a cell viability assay was conducted as previously described with modifications (Denizot, 1986, Journal Immunology. Methods, 89:271-277). On 96 well plates, cells of interest were seeded at 10,000 calls per well 48 hr prior to infection. Cells were then treated with virus alone, drug alone, or in combination. Cell viability was measured at different time points by removing the media, adding 50 ☒l of 1mg/ml solution of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-^{2}H-tetrazolium bromide) (Sigma, St. Louis, MO) and incubating for 3 hrs at 37°C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 50 ☒l of isopropanol followed by 30C incubation for 0.5 hr. The absorbency was determined on a microplate reader (Molecular Dynamics) at 560 nm (test wavelength) and 690 nm (reference wavelength). The percentage of surviving cells was estimated by dividing the OD_{550}-OD_{650} of virus or drug treated cells by the OD_{550}-OD_{650} of control cells. 6 replica samples were taken for each time point and each experiment was repeated at least three times.

For statistical analysis, CurveExpert (shareware by Daniel Hyams, version 1.34) was used to plot the dose-response curves for virus and drugs. Based upon the dose-response curves, the isobolograms were made according to the original theory of Steel and Peckham (1993, *Int. J. Rad. Onc. Biol. Phys.*, 5:85) and method described in Aoe et al. (1999, *Anticancer Res.* 19:291-299).

Animal studies. Six to eight week old athymic BALB/C *nu/nu* mice were obtained from Simonson Laboratories (Gilroy, California) and acclimated to

laboratory conditions one week prior to tumor implantation. Xenografts were established by injecting $1x10^6$ Hep3B, HepG2 or LNCaP cells suspended in 100⊠l of RPMI 1640 media subcutaneously. When tumors reached between 200 mm^3 and 300 mm^3, mice were randomized and dosed with 100 ⊠l of test article via intratumoral or the tail vein injection. Tumors were measured in two dimensions by external caliper and volume was estimated by the formula [length (mm) x width $(mm)^2$]/2. Animals were humanely killed when their tumor burden became excessive. Serum was harvested weekly by retro-orbital bleed. The level of AFP in the serum was determined by AFP Immunoassay kit (Genzyme Diagnostics, San Carlos, CA). The difference in mean tumor volume and mean serum AFP concentration between treatment groups was compared for statistical significance using the unpaired, two-tailed, *t*-test.

Transcription and Translation of E1A/E1B Bicistronic Cassette of CV890 in Different Cells.

In wild type adenovirus infection, E1A and E1B genes produce a family of alternatively spliced products. It has been found that there are five E1A mRNAs, among them 12S (880 nucleotides, nts) and 13S (1018 nts) mRNAs are the dominant ones that are expressed both early and late after infection. The 12S and 13S mRNAs encode the gene product of 243 amino acids (243R) and 289 amino acids (289R) respectively (reviewed by Shenk, 1996). The two major E1B transcripts that code for 19kD and 55kD proteins are 12S (1031 nts) and 22S (2287 nts) mRNAs. E1B 12S mRNA only codes the 19kD product, whereas the 22S mRNA codes for both 19kD and 55kD products due to different initiation sites during translation. In the current study, the generation of E1A-IRES-E1B bicistronic cassette was expected to change the pattern of E1A and E1B transcripts in viral infection. Therefore, Northern blot analysis was conducted to evaluate the steady-state level of E1A and E1B transcripts. First, CV802 or CV890 were infected to Hep3B (AFP) or HBL100 (AFP) cells for 24 hours. The total RNA samples were separated on agarose gels and processed for Northern blot by hybridizing to antisense RNA probes. The Northern blot with E1A

probe visualized the 12S and 13S mRNAs in both wild type CV802 infected cells. For CV890, E1A transcripts can only be seen in Hep3B cells, indicating the conditional transcription of ElA. It is of interest to find that in CV890, there is only one large transcript (about 3.51Kb), whereas the 12S and 13S mRNAs are no longer present. This large transcript indicates the continuous transcription of E1A-IRES-E1B bicistronic cassette, suggesting an alteration of viral E1A splicing pattern in CV890. Transcription of E1B from CV890 also appears to be AFP-dependent. It is clear that both 12S and 22S mRNAs of E1B were present in wild type CV802 samples, whereas the 128 mRNA and an enlarged 22S mRNA (3.5Kb) appeared in CV890 infected cells. Obviously, the identity of this enlarged mRNA is the same 3.5Kb transcript as visualized in E1A blot, which is from the transcription of E1A/E1B bicistronic cassette. Therefore, the E1B mRNA is tagged after E1A mRNA in this large transcript. This large transcript contains all the coding information for E1A, E1B 19kD and E1B 55kD. The mRNA splice pattern that appears in CV802 is not valid in CV890, the12S mRNA with E1B probe disappeared. Meanwhile, in the E1B Northern blot, due to the selection of our E1B probe (1540bp-3910bp), mRNA of the Adenovirus gene IX (3580bp-4070bp), the hexon-associated protein, was also detected. In CV890 infected Hep3B cells, gene IX expression is equivalent to that of CV802, whereas in CV890 infected HBL100, its expression was also completely shut down. This result further demonstrated that the AFP controlled E1A/E1B expression is the key for late gene expression as well as viral replication.

Results of the same samples in the Western blot also indicate that CV890 has AFP dependent expression of E1A and E1B. Under our experimental conditions, E1A expression level of CV890 in Hep3B cells is similar to that of CV802. However, when E1B 19kD protein was detected, it was found that the expression level was much lower than CV802 E1A. Previously, it has been addressed that IRES-mediated second gene has less expression (Mizuguchi et al., 2000, *Mol. Ther.* 1:376-382). Taken together, CV890 infection in permissive Hep3B cells can produce

normal amounts of E1A and lesser amounts of E1B proteins capable of initiating a normal productive infection. In AFP$^-$ cells, however, this process was attenuated due to a lack of E1A and E1B gene transcription and translation. These data demonstrated that the expression of both E1A and E1B genes are under the control of AFP TRE and the artificial E1A/E1B bicistronic cassette is functioning properly in CV890.

In Vitro Replication Specificity of CV890 in Tumor Cells and Primary Cells.

From *in vitro* comparison of virus yield, CV890 has a better specificity profile than CV732 (CV732 is an AFP-producing, cell-specific adenovirus variant in which the E1A gene is under control of AFP-TRE). In order to gain further insights of using CV890 in liver cancer therapy, more tumor cell lines and primary cells were tested to characterize *in vitro* virus replication. First, all cells in the study were analyzed for their AFP status by AFP immune assay. Based on AFP produced in the cells and media, all the cells were divided into three groups, high (>2.5 ☒g/10^6 cells/10 days), low (<0.6 ☒g/10^6 cells/10 days) and none (undetectable in our study) (Table 7). It was confirmed that replication of CV890 in different cell lines correlates well with the AFP status of the host cell. Among the group of liver cell lines, CV890 only replicates well in AFP$^+$ cells, including Hep3B, HepG2, Huh7, SNU449 and PLC/PRF/5. The amount of AFP required for the promoter activity seems very low as one of the hepatoma cell lines, SNU449, a previous reported AFP$^-$ cell (Park et al., 1995, *Int. J. Cancer* 62:276-282), produces very low AFP (about 60 ng/10^6 cells/10 days) compared to other cells. Nevertheless, even with very low amount of AFP, SNU449 cells can still support CV890 replication to the extent comparable to cells producing significantly higher levels of AFP such as HepG2. Compared to CV802, CV890 is attenuated 5,000 to 100,000 fold in cells that do not produce AFP, including the hepatoma cell Sk-Hepl and Chang liver cell, other tumor cells and primary cells. Taken together the results indicate that CV890 has shown a good

specificity profile from a broad spectrum of tumor cells. Among them, only the AFP^+ liver cells, AFP production level from high to low, are permissive for CV890.

In another experiment, CV890 was compared to CV802 for their single step growth curves on different cells. Results demonstrated that CV890 has a similar growth kinetics to wild-type CV802 in AFP^+ cells except that virus yields are slightly lower (2-8 fold) in low AFP producing cells. In consideration of experimental error, there is no dramatic difference in the replication of CV890 and CV802 in AFP^+ hepatoma cells. However, the growth curves of CV890 in AFP^- cells showed clear attenuation. During a 5 day experiment, CV890 failed to replicate in AFP^- cells including hepatoma cell (Change liver) and primary cells (nHLFC). From all the *in vitro* virus replication studies, it is clear that replication of CV890 is under the tight control of AFP-TRE and this adenovirus variant has an excellent specificity profile of preferentially targeting AFP producing hepatocellular carcinoma cells.

In Vivo Specificity and Efficacy of CV890.

CV890 specificity was also evaluated in animals bearing prostate cancer LNCaP xenografts. In this *in vivo* test, nude mice with prostate xenograft were intravenously injected with either CV890 or CV787, a prostate cancer specific adenovirus variant (Yu et al., 1999, Cancer Research, 59:4200-4203). Tumor volumes were documented and indicated that only CV787 had a significant antitumor efficacy in LNCaP xenografts, while tumors in the animals treated with CV890 grew, from 400 mm^3 to approximately 1200 mm^3 in six weeks, similar to the group treated with vehicle. This study indicates that CV890 does not attack LNCaP xenograft and keeps the good specificity profile under *in vivo* conditions.

To evaluate *in vivo* antitumor efficacy of CV890, different studies were carried out in the nude mouse model harboring human hepatoma xenografts. First, BALB/c nu/nu mice with HepG2 or Hep3B xenografts were established, animals were further challenged with single dose or multiple doses of CV890 into the tumor mass (intratumoral administration, IT) or via their tail vein (intravenous

administration, IV). Tumor volume and the level of serum AFP were monitored weekly after the start of treatment, and hence the efficacy of the treatment was determined. The *in vitro* cytotoxicity study has demonstrated that CV890 has a better cytolytic effect than CV732. In order to further examine their antitumor activity, we first conducted animal study to compare CV890 to CV732. Animals harboring 300 mm^3 Hep3B xenograft were grouped (n=6) and injected with vehicle alone (control group), CV890 ($1x10^{11}$ particles/dose, CV890 group), or CV732 ($1x10^{11}$ particles/dose, CV732 group). The Hep3B xenograft is a very aggressive tumor model and tumors grow very fast. Most animals can not survive long because of excessive tumor burden. During a six week study, single intravenous administration of CV890 have shown significant tumor growth inhibition, whereas control mice had over 10 fold tumor growth at week 5. In the group treated with CV732, single dose IV injection also reduced the tumor growth as compared to control group, however, it was much less effective compared to CV890. For example, the average tumor volume of the CV890 treated group dropped from 312 mm^3 to 219 mm^3, while tumor volume increased from 308 mm^3 to 1542 mm^3 5 weeks after treatment in control. Both control group and the CV732 group were terminated at week 5 because excessive tumor size. Previously, CV732 has been demonstrated to restrict the hepatoma tumor from growth after 5 doses of intravenous administration. Similar efficacy can be achieved with just a single intravenous administration of CV890, indicating that under *in vivo* conditions, CV890 has better efficacy than CV732 in hepatoma xenografts. In this experiment, 4 out of five CV890 treated mice were tumor free three weeks after treatment. However, in CV732 group, xenografts in two mice stopped growing but none of treated animals were tumor free through the six-week experiment. There was no tumor reduction in this group or the control group of animals. By statistical analysis, the differences in mean relative tumor volumes and serum AFP concentrations between CV890 treated and CV732 treated or vehicle treated tumors are significant ($p<0.01$)). Taken together, these studies suggest that

CV890 has a significant antitumor activity and its oncolytic efficacy is better than CV732, an adenovirus variant similar to AvE1a04I, in which the AFP TRE was applied to control E1A alone (Hallenback et al, 1999, Hum. Gene. Ther., 10:1721-1733).

Synergistic Antitumor Efficacy of CV890 in Combination with Chemotherapeutic Agents

In this example, different chemotherapeutic agents were tested in combination with CV890 for their *in vitro* killing effect in Hep3B or HepG2 cells. Drug concentrations were optimized to the extent that they would not generate extensive cytotoxic effect on their own. Under such conditions, some agents had shown higher cell killing effect in combination with CV890. Among them, doxorubicin, a drug currently used in treatment of HCC showed synergistic cytotoxicity with CV890. In experiments using doxorubicin together with CV890 on Hep3B cells, doxorubicin at 10ng/ml did not generate cytotoxicity, whereas CV890 at an MOI of 0.01 (pfu/cell) only had about 35% of cell killed at day 9. However, when both were applied together, 90% cells were killed 9 days after treatment. In order to determine the potential synergistic effect from the combination treatment, the MTT cell viability data were subjected to further statistical analysis. Figure 10 shows a representative IC_{50} isobologram of doxorubicin and CV890 on Hep3B cells at day 5. First, the dose-response curves of doxorubicin alone or CV890 alone were made. Based on the original theory of Steel and Peckham (1993) and method by Aoe et al. (1999), three isoeffect curves (mode I and mode 2a, 2b) were constructed. From this isobologram, several data points were in the synergy or additive area, indicating that combination of CV890 and doxorubicin provides synergistic effect on killing of Hep3B cells.

Although CV890 alone has good antitumor activity, we applied combination therapy with doxorubicin for *in vivo* evaluation of synergy. Animals harboring 300 mm^3 Hep3B xenografts were grouped (n=6) and injected with vehicle alone (control group), CV890 alone ($1x10^{11}$ particles/dose, CV890 group), doxorubicin alone

(10mg/kg, doxorubicin group), or CV890 in combination with doxorubicin (combination group). Figure 11 shows weekly change of the relative tumor size normalized to 100% at day 1. In this experiment, by week six, all animals in the control group had excessive tumor which has increased by 700% of baseline, whereas in CV890 group and combination group, animals had either tumor free or tumor reduction. Of the eight Hep3B xenografts, treated with CV890, three animals (37.5%) had no palpable tumor at week 5, another three animals had tumor regressed by more than 60%. In combination group, four out of eight animals were tumor free from week 5, another four animals had tumor reduction about 90%. All the animals in the CV890 and combination group were alive and tumor was suppressed even ten weeks following treatment whereas the control animals were sacrificed for excessive tumor burden after week 6. Furthermore, CV890 also caused a drop in the serum AFP concentration in these mice. Statistical analysis shows that differences in mean relative tumor volumes and serum AFP concentrations between CV890 and vehicle treated group or combination and doxorubicin treated group are significant at different times ($p<0.005$).

The strong efficacy in the combination treatment shows that single IV injection of CV890 in combination of doxorubicin can eradicate aggressive Hep3B xenografts in most of the animals.

Table 7. AFP production in different tumor cells

AFP

CELLS	(ng/10^6cells/10days)	
Hep3B	2645	High
HepG2	3140	
HuH7	4585	
SNU449	60	Low
PLC/PRF/5	600	
Chang	0	None
SK-Hep1	0	
HBL100	0	
PA-1	0	
LoVo	0	

TABLE 1 IRES SEQUENCES

SEQ ID NO:1 A 519 base pair IRES obtainable from encephelomycarditis virus (EMCV).

```
1     GACGTCGACTAATTCCGGTTATTTTCCACCATATTGCCGTCTTTTGGCAA
          SalI
51    TGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGG
101   GTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAG
151   GAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGAC
201   CCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCC
251   AAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGTGC
301   CACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAG
351   CGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGG
401   GATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGG
451   TTAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGA
                    SalI
501   AAAACACGATGTCGACGTC
```

SEQ ID NO:2 An IRES obtainable from vascular endothelial growth factor (VEGF).

```
1     ACGTAGTCGACAGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCAGGCCC
          SalI
51    CGGCCCGGGCCTCGGTTCCAGAAGGGAGAGGAGCCCGCCAAGGCGCGCAA
101   GAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGCCGCGC
151   CGGCCCCGGACGGCCTCCGAAACCATGGTCGACACGTA
                                      SalI
```

SEQ ID NO:3 A 5'UTR region of HCV.

```
1    GCCAGCCCCCTGATGGGGGCGACACTCCGCCATGAATCACTCCCCTGTGAGGAACTACTG
61   TCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGAC
121  CCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAG
181  GACGACCGGGTCCTTTCTTGGATTAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCC
241  GCAAGACTGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG
301  GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACC (341)
```

SEQ ID NO:4A 5'UTR region of BiP SEQ ID NO:4

```
1    CCCGGGGTCACTCCTGCTGGACCTACTCCGACCCCCTAGGCCGGGAGTGAAGGCGGGACT
61   TGTGCGGTTACCAGCGGAAATGCCTCGGGGTCAGAAGTCGCAGGAGAGATAGACAGCTGC
121  TGAACCAATGGGACCAGCGGATGGGGCGGATGTTATCTACCATTGGTGAACGTTAGAAAC
181  GAATAGCAGCCAATGAATCAGCTGGGGGGGCGGAGCAGTGACGTTTATTGCGGAGGGGGC
241  CGCTTCGAATCGGCGGCGGCCAGCTTGGTGGCCTGGGCCAATGAACGGCCTCCAACGAGC
301  AGGGCCTTCACCAATCGGCGGCCTCCACGACGGGGCTGGGGGAGGGTATATAAGCCGAGT
361  AGGCGACGGTGAGGTCGACGCCGGCCAAGACAGCACAGACAGATTGACCTATTGGGGTGT
421  TTCGCGAGTGTGAGAGGGAAGCGCCGCGGCCTGTATTTCTAGACCTGCCCTTCGCCTGGT
481  TCGTGGCGCCTTGTGACCCCGGGCCCCTGCCGCCTGCAAGTCGAAATTGCGCTGTGCTCC
541  TGTGCTACGGCCTGTGGCTGGACTGCCTGCTGCTGCCCAACTGGCTGGCAAGATG (595)
```

SEQ ID NO:5 A 5'UTR of PDGF SEQ ID NO:5

```
1    GTTTGCACCTCTCCCTGCCCGGGTGCTCGAGCTGCCGTTGCAAAGCCAACTTTGGAAAAA
61   GTTTTTTGGGGGAGACTTGGGCCTTGAGGTGCCCAGCTCCGCGCTTTCCGATTTTGGGGG
121  CTTTCCAGAAAATGTTGCAAAAAAGCTAAGCCGGCGGGCAGAGGAAAACGCCTGTAGCCG
181  GCGAGTGAAGACGAACCATCGACTGCCGTGTTCCTTTTCCTCTTGGAGGTTGGAGTCCCC
241  TGGGCGCCCCCACACCCCTAGACGCCTCGGCTGGTTCGCGACGCAGCCCCCCGGCCGTGG
301  ATGCTGCACTCGGGCTCGGGATCCGCCCAGGTAGCCGGCCTCGGACCCAGGTCCTGCGCC
361  CAGGTCCTCCCCTGCCCCCCAGCGACGGAGCCGGGGCCGGGGGCGGCGGCGCCGGGGGCA
421  TGCGGGTGAGCCGCGGCTGCAGAGGCCTGAGCGCCTGATCGCCGCGGACCTGAGCCGAGC
481  CCACCCCCCTCCCCAGCCCCCCACCCTGGCCGCGGGGGCGGCGCGCTCGATCTACGCGTC
541  CGGGGCCCCGCGGGGCCGGGCCCGGAGTCGGCATG (575)
```

TABLE 2 LITERATURE REFERENCES FOR IRES

IRES Host	Example	Reference
Picornavirus	HAV	Glass et al., 1993. Virol 193:842-852
	EMCV	Jang & Wimmer, 1990. Gene Dev 4:1560-1572
	Poliovirus	Borman et al., 1994. EMBO J 13:3149-3157
HCV and pestivirus	HCV	Tsukiyama-Kohara et al., 1992. J Virol 66:1476-1483
	BVDV	Frolov I et al., 1998. RNA. 4:1418-1435
Leishmania virus	LRV-1	Maga et al., 1995. Mol Cell Biol 15:4884-4889
Retroviruses	MoMLV VL30 (Harvey murine sarcoma virus)	Torrent et al., 1996. Hum Gene Ther 7:603-612
	REV	Lopez-Lastra et al., 1997. Hum Gene Ther 8:1855-1865
Eukaryotic mRNA	BiP	Macejak & Sarnow, 1991. Nature 353:90-94
	antennapedia mRNA	Oh et al., 1992. Gene & Dev 6:1643-1653
	FGF-2	Vagner et al., 1995. Mol Cell Biol 15:35-44
	PDGF-B	Bernstein et al., 1997. J Biol Chem 272:9356-9362
	IGFII	Teerink et al., 1995. Biochim Biophys Acta 1264:403-408
	eIF4G	Gan & Rhoads, 1996. J Biol Chem 271:623-626
	VEGF	Stein et al., 1998. Mol Cell Biol 18:3112-3119; Huez et al., 1998. Mol Cell Biol 18:6178-6190

TABLE 3 TRE SEQUENCES

Nucleotide sequence of a human uroplakin II 5' flanking region. Position +1 (the translational start site) is denoted with an asterisk. SEQ ID NO:6 (number 1 of SEQ ID NO:6 corresponds to position -2239 with respect to the translational start site).

```
TCGATAGGTA  CCCACTATAG  GGCACGCGTG  GTCGACGGCC  CGGGCTGGTC
1                                                       50

TGGCAACTTC  AAGTGTGGGC  CTTTCAGACC  GGCATCATCA  GTGTTACGGG
51                                                     100

GAAGTCACTA  GGAATGCAGA  ATTGATTGAG  CACGGTGGCT  CACACCTGTA
101                                                    150

ATCCCAACAC  TCTGGGAGGC  CAAGGCAGGT  GGATCACTTG  TGGTCAGGAG
151                                                    200

TTTGAGACCA  GCCTGGCCAA  CATGGTGAAA  CCTCATCTCT  ACTAAAAATA
201                                                    250

CAAAAATTAG  CTGGGAATGG  TGGCACATGC  CTATAATCCC  AGTTACTCAG
251                                                    300

GAGGCTGAGG  CAGGAGAATC  ATTTGAACCT  GGGAGGCAGA  GGTTGCAGTG
301                                                    350

AGCCGAGATC  ACGCCACTGC  ACTCCAGCCT  GGGTGACACA  GCGAGACTCT
351                                                    400

GTCTCAAAAA  AAAAAAAATG  CAGAATTTCA  GGCTTCACCC  CAGACCCACT
401                                                    450

GCATGACTGC  ATGAGAAGCT  GCATCTTAAC  AAGATCCCTG  GTAATTCATA
451                                                    500

CGCATATTAA  ATTTGGAGAT  GCACTGGCGT  AAGACCCTCC  TACTCTCTGC
501                                                    550

TTAGGCCCAT  GAGTTCTTCC  TTTACTGTCA  TTCTCCACTC  ACCCCAAACT
551                                                    600

TTGAGCCTAC  CCTTCCCACC  TTGGCGGTAA  GGACACAACC  TCCCTCACAT
601                                                    650
```

TCCTACCAGG ACCCTAAGCT TCCCTGGGAC TGAGGAAGAT AGAATAGTTC
651 700

GTGGAGCAAA CAGATATACA GCAACAGTCT CTGTACAGCT CTCAGGCTTC
701 750

TGGAAGTTCT ACAGCCTCTC CCGACAAAGT ATTCCACTTT CCACAAGTAA
751 800

CTCTATGTGT CTGAGTCTCA GTTTCCACTT TTCTCTCTCT CTCTCTCTCT
801 850

CAACTTTCTG AGACAGAGTT TCACTTAGTC GCCCAGGCTG GAGTGCAGGG
851 900

GCACAATCTC GGCTCACTGC AACCTCCACC TCCTGGGTTC AAGTGTTTCT
901 950

CCTGTCTCAG CCTCCCGAGT AGCTGGGATT ACAGGCACAC ACCACCGCGT
951 1000

TAGTTTTTGT ATTTTTGGTA GAGATGGTGT TTCGCCATAT TGGCCAGGCT
1001 1050

GATCTCGAAC TCCTGACCTC AGGTGATCCG CCCACCTCGG CCTCCCAAAG
1051 1100

TGCTGGGATT ACAGGCATGA GCCACCACGC CCGGCTGATC TCTTTTCTAT
1101 1150

TTTAATAGAG ATCAAACTCT CTGTGTTGCC TAGGCTGGTC TTGAACTCCT
1151 1200

GGCCTCGAGT GATCCTCCCA CCTTGGCCTC CCAAAGTGTT GAGATTACAG
1201 1250

GCATGAGCCA CTGTGCCTGG CCTCAGTTCT ACTACAAAAG GAAGCCAGTA
1251 1300

CCAGCTACCA CCCAGGGTGG CTGTAGGGCT ACAATGGAGC ACACAGAACC
1301 1350

CCTACCCAGG GCCCGGAAGA AGCCCCGACT CCTCTCCCCT CCCTCTGCCC
1351 1400

AGAACTCCTC CGCTTCTTTC TGATGTAGCC CAGGGCCGGA GGAGGCAGTC

1401 1450

AGGGAAGTTC TGTCTCTTTT TCATGTTATC TTACGAGGTC TCTTTTCTCC
1451 1500

ATTCTCAGTC CAACAAATGG TTGCTGCCCA AGGCTGACTG TGCCCACCCC
1501 1550

CAACCCCTGC TGGCCAGGGT CAATGTCTGT CTCTCTGGTC TCTCCAGAAG
1551 1600

TCTTCCATGG CCACCTTCGT CCCCACCCTC CAGAGGAATC TGAAACCGCA
1601 1650

TGTGCTCCCT GGCCCCCACA GCCCCTGCCT CTCCCAGAGC AGCAGTACCT
1651 1700

AAGCCTCAGT GCACTCCAAG AATTGAAACC CTCAGTCTGC TGCCCCTCCC
1701 1750

CACCAGAATG TTTCTCTCCC ATTCTTACCC ACTCAAGGCC CTTTCAGTAG
1751 1800

CCCCTTGGAG TATTCTCTTC CTACATATCA GGGCAACTTC CAAACTCATC
1801 1850

ACCCTTCTGA GGGGTGGGGG AAAGACCCCC ACCACATCGG GGGAGCAGTC
1851 1900

CTCCAAGGAC TGGCCAGTCT CCAGATGCCC GTGCACACAG GAACACTGCC
1901 1950

TTATGCACGG GAGTCCCAGA AGAAGGGGTG ATTTCTTTCC CCACCTTAGT
1951 2000

TACACCATCA AGACCCAGCC AGGGCATCCC CCCTCCTGGC CTGAGGGCCA
2001 2050

GCTCCCCATC CTGAAAAACC TGTCTGCTCT CCCCACCCCT TTGAGGCTAT
2051 2100

AGGGCCCAAG GGGCAGGTTG GACTGGATTC CCCTCCAGCC CCTCCCGCCC
2101 2150

CCAGGACAAA ATCAGCCACC CCAGGGGCAG GGCCTCACTT GCCTCAGGAA
2151 2200

```
CCCCAGCCTG  CCAGCACCTA  TTCCACCTCC  CAGCCCAGCA
2201                                        2239
```

Nucleotide sequence of a mouse uroplakin II 5' flanking region. The translational start site is denoted with an asterisk. SEQ ID NO:7 (number 1 of SEQ ID NO:7 corresponds to position -3592 with respect to the translational start site).

```
CTCGAGGATCTCGGCCCTCTTTCTGCATCCTTGTCCTAAATCATTTTCAT
1                                                  50

ATCTTGCTAGACCTCAGTTTGAGAGAAACGAACCTTCTCATTTTCAAGTT
51                                                 100

GAAAAAAAAAAGAGGTTCAAAGTGGCTCACTCAAAGTTACAAGCCAACAC
101                                                150

TCACCACTACGAGTACAATGGCCACCATTAGTGCTGGCATGCCCCAGGAG
151                                                200

ACAGGCATGCATATTATTCTAGATGACTGGGAGGCAGAGGGGTGGCCTAG
201                                                250

TGAGGTCAGACTGTGGACAGATCAGGCAGATGTGGGTTCTGATCCCAATT
251                                                300

CCTCAGGCCGCAGAACTACTGTGGTTCAAGAAGGGGACAAAAGGACTGCA
301                                                350

GTCCGGAACAGGAGGTCCATTTGAGAGCTGACTGAGCAGAAGAGGAAAGT
351                                                400

GAAGAACTTCTGGGGCAAGAGCTTACCCTACTTTACAGCTTTGTTGTCTT
401                                                450

CTTTACTCCAGGGGCGTCCCTGGTACTCAGTAAATGTCTGTTGGCTTGAG
451                                                500

GAACATATGTGTAAGGAGGAAGGAGAGGGAACTTGAGGGAGTTAAGACTC
501                                                550
```

AAGAATCAATCAAGGAGAGGACAGCAGAGAAGACAGGGTTTGGGAGAGAG
551 600

ACTCCAGACATTGGCCCTGGTTCCCTTCTTGGCCACTGTGAAACCCTCCA
601 650

GAGGAACTGAGTGCTGTGGCTTTAAATGATCTCAGCACTGTCAGTGAAGC
651 700

GCTCTGCTCAAAGAGTTATCCTCTTGCTCCTGTGCCGGGGCCTCCCCCTC
701 750

CTCTCAGCTCCCAAACCCTTCTCAGCCACTGTGATGGCATAATTAGATGC
751 800

GAGAGCTCAGACCGTCAGGTCTGCTCCAGGAACCACCCATTTTCCCCAAC
801 850

CCCAGAGAAAGGTCCTAGTGGAAAAGTGGGGGCCACTGAAGGGCTGATGG
851 900

GGTTCTGTCCTTTCCCCCATGCTGGGTGGACTTAAAGTCTGCGATGTGTG
900 950

TAGGGGGTAGAAGACAACAGAACCTGGGGGCTCCGGCTGGGAGCAGGAGG
951 1000

AACTCTCACCAGACGATCTCCAAATTTACTGTGCAATGGACGATCAGGAA
1001 1050

ACTGGTTCAGATGTAGCTTCTGATACAGTGGGTCTGAGGTAAAACCCGAA
1051 1100

ACTTAATTTCTTTCAAAAATTTAAAGTTGCATTTATTATTTTATATGTGT
1101 1150

GCCCATATGTGTGCCACAGTGTCTATGTGGAGGTCAGAGGGCAAGTTGTG
1151 1200

GGCATTGGCTCTCTCCTTTCATAATGTGGCTTCTGGGGACCAAAATGTCA
1201 1250

GGCATGGTGGCAAGAGCTTTTACCTGTTGAGCCATCTCATGGTTTCGTAA

1251 1300

AACTTCCTATGACGCTTACAGGTAACGCAGAGACACAGACTCACATTTGG
1301 1350

AGTTAGCAGATGCTGTATTGGTGTAAACACTCATACACAGACACACACAC
1351 1400

ATACTCATACACACACACACACTTATCACATGCACACACATACTCGTA
1401 1450

TACACACAGACACACACACATGCACTCTCACATTCACATATTCATACACA
1451 1500

TCCACACACACACTCATCCACACACACAGACACACATACTCATCCACACA
1501 1550

CACACACACACATACTCATACACACACACAGACACACATACTCATACACA
1551 1600

CACACAGACACACACATATAATCATACATACACAGACACACTCATACATG
1601 1650

TGCACACACACACTCATCCACACACACACACTCATACACACACACACTCA
1651 1700

TACACACACACACTCATACACACACACACGAGGTTTTTCTCAGGCTGCCT
1701 1750

TTGGGTGGAGACTGGAACTGATTTCTGTTTTTCAGCTCCTTGGCTTTTTG
1751 1800

TCCCTTTAGATGAGATCTCCTCCTCACTTTACACACAGAAAGATCACACA
1801 1850

CGAGGGAGAACTGGCGGTGCGGAAGAGGGCTACACGGTAGGGTGTCAGGG
1851 1900

TCAGGAGATCTTCCTGGCAAGTCTCAAACCTCCACATAGCACAGTGTTTA
1901 1950

CGTGAGGATTTAGGAGGAATCAGGAAGAGGATTGGTTTACTGCAGAGCAG
1951 2000

ACCATATAGGTCCACTCCTAAGCCCCATTTGAAATTAGAAGTGAGACAGT
2001 2050

GTGGGATAAAAAGAGCAGATCTCTGGTCACATTTTTAAAGGGATATGAGG
2051 3000

GTCCTGTGCCTTTAAGCCTTCCCATCTCCCTCCAATCCCCCCTCACCTTC
2101 2150

CCCACCCTAACCCTCCCCAGGTTTCTGGAGGAGCAGAGTTGCGTCTTCTC
2151 2200

CCTGCCCTGCCGAGCTGCTCACTGGCTGCTCTAGAGGCTGTGCTTTGCGG
2201 2250

TCTCCATGGAAACCATTAGTTGCTAAGCAACTGGAGCATCATCTGTGCTG
2251 2300

AGCTCAGGTCCTATCGAGTTCACCTAGCTGAGACACCCACGCCCCTGCAG
2301 2350

CCACTTTGCAGTGACAAGCCTGAGTCTCAGGTTCTGCATCTATAAAAACG
2351 2400

AGTAGCCTTTCAGGAGGGCATGCAGAGCCCCCTGGCCAGCGTCTAGAGGA
2401 2450

GAGGTGACTGAGTGGGGCCATGTCACTCGTCCATGGCTGGAGAACCTCCA
2451 2500

TCAGTCTCCCAGTTAGCCTGGGGCAGGAGAGAACCAGAGGAGCTGTGGCT
2501 2550

GCTGATTGGATGATTTACGTACCCAATCTGTTGTCCCAGGCATCGAACCC
2551 2600

CAGAGCGACCTGCACACATGCCACCGCTGCCCCGCCCTCCACCTCCTCTG
2601 2650

CTCCTGGTTACAGGATTGTTTTGTCTTGAAGGGTTTTGTTGTTGCTACTT
2651 2700

TTTGCTTTGTTTTTTCTTTTTTAACATAAGGTTTCTCTGTGTAGCCCTAG
2701 2750

CTGTCCTGGAACTCACTCTGTAGACCAGGCTGGCCTCAAACTCAGAAATC
2751 2800

CACCTTCCTCCCAAGTGCTGGGATTAAAGGCATTCGCACCATCGCCCAGC
2801 2850

CCCCGGTCTTGTTTCCTAAGGTTTTCCTGCTTTACTCGCTACCCGTTGCA
2851 2900

CAACCGCTTGCTGTCCAAGTCTGTTTGTATCTACTCCACCGCCCACTAGC
2901 2950

CTTGCTGGACTGGACCTACGTTTACCTGGAAGCCTTCACTAACTTCCCTT
2951 3000

GTCTCCACCTTCTGGAGAAATCTGAAGGCTCACACTGATACCCTCCGCTT
3001 3050

CTCCCAGAGTCGCAGTTTCTTAGGCCTCAGTTAAATACCAGAATTGGATC
3051 3100

TCAGGCTCTGCTATCCCCACCCTACCTAACCAACCCCCTCCTCTCCCATC
3101 3150

CTTACTAGCCAAAGCCCTTTCAACCCTTGGGGCTTTTCCTACACCTACAC
3151 3200

ACCAGGGCAATTTTAGAACTCATGGCTCTCCTAGAAAACGCCTACCTCCT
3201 3250

TGGAGACTGACCCTCTACAGTCCAGGAGGCAGACACTCAGACAGAGGAAC
3251 3300

TCTGTCCTTCAGTCGCGGGAGTTCCAGAAAGAGCCATACTCCCCTGCAGA
3301 3350

GCTAACTAAGCTGCCAGGACCCAGCCAGAGCATCCCCCTTTAGCCGAGGG
3351 3400

CCAGCTCCCCAGAATGAAAAACCTGTCTGGGGCCCCTCCCTGAGGCTACA

3401 3450

GTCGCCAAGGGGCAAGTTGGACTGGATTCCCAGCAGCCCCTCCCACTCCG

3451 3500

AGACAAAATCAGCTACCCTGGGGCAGGCCTCATTGGCCCCAGGAAACCCC

3501 3550

AGCCTGTCAGCACCTGTTCCAGGATCCAGTCCCAGCGCAGTA

3551 3592

AFP-TRE. SEQ ID NO:8.

```
1    GCATTGCTGTGAACTCTGTACTTAGGACTAAACTTTGAGCAATAACACACATAGATTGAG

61   GATTGTTTGCTGTTAGCATACAAACTCTGGTTCAAAGCTCCTCTTTATTGCTTGTCTTGG

121  AAAATTTGCTGTTCTTCATGGTTTCTCTTTTCACTGCTATCTATTTTTCTCAACCACTCA
181  CATGGCTACAATAACTGTCTGCAAGCTTATGATTCCCAAATATCTATCTCTAGCCTCAAT

241  CTTGTTCCAGAAGATAAAAAGTAGTATTCAAATGCACATCAACGTCTCCACTTGGAGGGC

301  TTAAAGACGTTTCAACATACAAACCGGGGAGTTTTGCCTGGAATGTTTCCTAAAATGTGT

361  CCTGTAGCACATAGGGTCCTCTTGTTCCTTAAAATCTAATTACTTTTAGCCCAGTGCTCA

421  TCCCACCTATGGGGAGATGAGAGTGAAAAGGGAGCCTGATTAATAATTACACTAAGTCAA

481  TAGGCATAGAGCCAGGACTGTTTGGGTAAACTGGTCACTTTATCTTAAACTAAATATATC

541  CAAAACTGAACATGTACTTAGTTACTAAGTCTTTGACTTTATCTCATTCATACCACTCAG

601  CTTTATCCAGGCCACTTATGAGCTCTGTGTCCTTGAACATAAAATACAAATAACCGCTAT

661  GCTGTTAATTATTGGCAAATGTCCCATTTTCAACCTAAGGAAATACCATAAAGTAACAGA

721  TATACCAACAAAAGGTTACTAGTTAACAGGCATTGCCTGAAAAGAGTATAAAAGAATTTC

781  AGCATGATTTTCCATATTGTGCTTCCACCACTGCCAATAACA (822)
```

Probasin-TRE SEQ ID NO:9

-426
5'-AAGCTTCCACAAGTGCATTTAGCCTCTCCAGTATTGCTGATGAATCCACAGT

TCAGGTTCAATGGCGTTCAAAACTTGATCAAAAATGACCAGACTTTATATTTA

CACCAACATCTATCTGATTGGAGGAATGGATAATAGTCATCATGTTTAAACAT

CTACCATTCCAGTTAAGAAAATATGATAGCATCTTGTTCTTAGTCTTTTTCTTA
ARE-1
ATAGGGACATAAAGCCCACAAATAAAAATATGCCTGAAGAATGGGACAGGC

ATTGGGCATTGTCCATGCCTAGTAAAGTACTCCAAGAACCTATTTGTATACTA
ARE-2
GATGACACAATGTCAATGTCTGTGTACAACTGCCAACTGGGATGCAAGACAC

TGCCCATGCCAATCATCCTGAAAAGCAGCTATAAAAAGCAGGAAGCTACTCT
CAAT box TATAA box

+1 +28
GCACCTTGTCAGTAGGTCCAGATACCTACAG-3'
Transcription site

Tyrosinase-TRE. SEQ ID NO:10

PinAl end

1 *CCGGT*TGAAAATGATAAGTTGAATTCTGTCTTCGAGAACATAGAAAAGAA

51 TTATGAAATGCCAACATGTGGTTACAAGTAATGCAGACCCAAGGCTCCCC

101 AGGGACAAGAAGTCTTGTGTTAACTCTTTGTGGCTCTGAAAGAAAGAGAG

151 AGAGAAAAGATTAAGCCTCCTTGTGGAGATCATGTGATGACTTCCTGATT

201 CCAGCCAGAGCGAGCATTTCCATGGAAACTTCTCTTCCTCTTCACTCGAG

251 ATTACTAACCTTATTGTTAATATTCTAACCATAAGAATTAAACTATTAAT

301 GGTGAATAGAGTTTTTCACTTTAACATAGGCCTATCCCACTGGTGGGATA

351 CGAGCCAATTCGAAAGAAAAAGTCAGTCATGTGCTTTTCAGAGGATGAAA

401 GCTTAAGATAAAGACTAAAAGTGTTTGATGCTGGAGGTGGGAGTGGTATT

451 ATATAGGTCTCAGCCAAGACATGTGATAATCACTGTAGTAGTAGCTGGAA

501 AGAGAAATCTGTGACTCCAATTAGCCAGTTCCTGCAGACCTTGTGA

PinAl end

Human glandular kallikrein-TRE SEQ ID NO:11

gaattcagaa ataggggaag gttgaggaag gacactgaac tcaaagggga tacagtgatt 60

ggtttatttg tcttctcttc acaacattgg tgctggagga attcccaccc tgaggttatg 120

aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agtttctcag 180

ccacagagat tcacagccta gggcaggagg acactgtacg ccaggcagaa tgacatggga 240

attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgggctt tgtagtaaca 300

agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctgtt acaaattttt 360

caagctggca gggaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaac 420

tgataaagga aatagccagg tgggggcctt tcccattgta ggggggacat atctggcaat 480

agaagccttt gagacccttt agggtacaag tactgaggca gcaaataaaa tgaaatctta 540

tttttcaact ttatactgca tgggtgtgaa gatatatttg tttctgtaca gggggtgagg 600

gaaaggaggg gaggaggaaa gttcctgcag gtctggtttg gtcttgtgat ccaggggtc 660

ttggaactat ttaaattaaa ttaaattaaa acaagcgact gttttaaatt aaattaaatt 720

aaattaaatt ttactttatt ttatcttaag ttctgggcta catgtgcagg acgtgcagct 780

ttgttacata ggtaaacgtg tgccatggtg gtttgctgta cctatcaacc catcacctag 840

gtattaagcc cagcatgcat tagctgtttt tcctgacgct ctccctctcc ctgactccca 900

caacaggccc cagtgtgtgt tgttcccctc cctgtgtcca tgtgttctca ttgttcagct 960

cccacttata agtgagaaca tgtggtgttt ggttttctgt ttctgtgtta gtttgctgag 1020

gataatggct tccacctcca tccatgttcc tgcaaaggac gtgatcttat tctttttat
1080

ggttgcatag aaattgtttt tacaaatcca attgatattg tatttaatta caagttaatc
1140

taattagcat actagaagag attacagaag atattaggta cattgaatga ggaaatatat
1200

aaaataggac gaaggtgaaa tattaggtag gaaaagtata atagttgaaa gaagtaaaaa
1260

aaaatatgca tgagtagcag aatgtaaaag aggtgaagaa cgtaatagtg acttttaga
1320

ccagattgaa ggacagagac agaaaaattt taaggaattg ctaaaccatg tgagtgttag
1380

aagtacagtc aataacatta aagcctcagg aggagaaaag aataggaaag gaggaaatat
1440

gtgaataaat agtagagaca tgtttgatgg attttaaaat atttgaaaga cctcacatca
1500

aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaagt
1560

tagaaatatt attggcaaag cttaaatgtt aaaagtccta gagagaaagg atggcagaaa
1620

tattggcggg aaagaatgca gaacctagaa tataaattca tcccaacagt ttggtagtgt
1680

gcagctgtag ccttttctag ataatacact attgtcatac atcgcttaag cgagtgtaaa
1740

atggtctcct cactttattt atttatatat ttatttagtt ttgagatgga gcctcgctct
1800

gtctcctagg ctggagtgca atagtgcgat accactcact gcaacctctg cctcctctgt
1860

tcaagtgatt ttcttacctc agcctcccga gtagctggga ttacaggtgc gtgccaccac
1920

acccggctaa tttttgtatt ttttgtagag acggggtttt gccatgttgg ccaggctggt
1980

cttgaactcc tgacatcagg tgatccacct gccttggcct cctaaagtgc tgggattaca
2040

ggcatgagcc accgtgccca accactttat ttatttttta tttttatttt taaatttcag
2100

cttctatttg aaatacaggg ggcacatata taggattgtt acatgggtat attgaactca
2160

ggtagtgatc atactaccca acaggtaggt tttcaaccca ctccccctct tttcctcccc
2220

attctagtag tgtgcagtgt ctattgttct catgtttatg tctatgtgtg ctccaggttt
2280

agctcccacc tgtaagtgag aacgtgtggt atttgatttt ctgtccctgt gttaattcac
2340

ttaggattat ggcttccagc tccattcata ttgctgtaaa ggatatgatt catttttcat
2400

ggccatgcag tattccatat tgcgtataga tcacattttc tttctttttt ttttttgaga
2460

cggagtcttg ctttgctgcc taggctggag tgcagtagca cgatctcggc tcactgcaag
2520

cttcacctcc ggggttcacg tcattcttct gtctcagctt cccaagtagc tgggactaca
2580

ggcgcccgcc accacgtccg gctaattttt ttgtgtgttt ttagtagaga tgggggtttc
2640

actgtgttag ccaggatggt cttgatctcc tgaccttgtg gtccacctgc ctcggtctcc
2700

caaagtgctg ggattacagg ggtgagccac tgcgcccggc ccatatatac cacattttct
2760

ttaaccaatc caccattgat gggcaactag gtagattcca tggattccac agttttgcta
2820

ttgtgtgcag tgtggcagta gacatatgaa tgaatgtgtc tttttggtat aatgatttgc
2880

attcctttgg gtatacagtc attaatagga gtgctgggtt gaacggtggc tctgtttaaa
2940

attctttgag aattttccaa actgtttgcc atagagagca aactaattta catttccacg
3000

aacagtatat aagcattccc ttttctccac agctttgtca tcatggtttt tttttttctt
3060

tattttaaaa aagaatatgt tgttgttttc ccagggtaca tgtgcaggat gtgcaggttt
3120

gttacatagg tagtaaacgt gagccatggt ggtttgctgc acctgtcaac ccattacctg
3180

ggtatgaagc cctgcctgca ttagctcttt tccctaatgc tctcactact gccccaccct
3240

caccctgaca gggcaaacag acaacctaca gaatgggagg aaatttttgc aatctattca
3300

tctgacaaag gtcaagaata tccagaatct acaaggaact taagcaaatt tttacttttt
3360

aataatagcc actctgactg gcgtgaaatg gtatctcatt gtggttttca tttgaatttc
3420

tctgatgatc agtgacgatg agcatttttt catatttgtt ggctgcttgt acgtcttttg
3480

agaagtgtct cttcatgcct tttggccact ttaatgggat tatttttgc tttttagttt
3540

aagttcctta tagattctgg atattagact tcttattgga tgcatagttt gtgaatactc
3600

tcttccattc tgtaggttgt ctgtttactc tattgatggc ttcttttgct gtgccgaagc
3660

atcttagttt aattagaaac cacctgccaa tttttgtttt tgttgcaatt gcttttgggg
3720

acttagtcat aaactctttg ccaaggtctg ggtcaagaag agtatttcct aggttttctt
3780

ctagaatttt gaaagtctga atgtaaacat ttgcattttt aatgcatctt gagttagttt
3840

ttgtatatgt gaaaggtcta ctctcatttt ctttccctct ttctttcttt ctttcttttc
3900

tttctttctt tctttctttc tttctttctt tctttctttc tttctttttg tccttctttc
3960

tttctttctt tctctttctt tctctctttc ttttttttt ttgatggagt attgctctgt
4020

tgcccaggct gcagtgcagc ggcacgatct cggctcactg caacctctgc ctcctgggtt
4080

caactgattc tcctgcatca gccttccaag tagctgggat tataggcgcc cgccaccacg
4140

cccgactaat ttttgtattt ttagtagaga cggggttgtg ccatgttggc caggctggtt
4200

tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgct gggattacag
4260

gtgtgagcca ctgtgcccag ccaagaatgt cattttctaa gaggtccaag aacctcaaga
4320

tattttggga ccttgagaag agaggaattc atacaggtat tacaagcaca gcctaatggc
4380

aaatctttgg catggcttgg cttcaagact ttaggctctt aaaagtcgaa tccaaaaatt
4440

tttataaaag ctccagctaa gctaccttaa aaggggcctg tatggctgat cactcttctt
4500

gctatacttt acacaaataa acaggccaaa tataatgagg ccaaaattta ttttgcaaat
4560

aaattggtcc tgctatgatt tactcttggt aagaacaggg aaaatagaga aaaatttaga
4620

ttgcatctga ccttttttc tgaattttta tatgtgccta caatttgagc taaatcctga
4680

attattttct ggttgcaaaa actctctaaa gaagaacttg gttttcattg tcttcgtgac
4740

acatttatct ggctctttac tagaacagct ttcttgtttt tggtgttcta gcttgtgtgc
4800

cttacagttc tactcttcaa attattgtta tgtgtatctc atagttttcc ttcttttgag
4860

aaaactgaag ccatggtatt ctgaggacta gagatgactc aacagagctg gtgaatctcc
4920

tcatatgcaa tccactgggc tcgatctgct tcaaattgct gatgcactgc tgctaaagct
4980

atacatttaa aaccctcact aaaggatcag ggaccatcat ggaagaggag gaaacatgaa
5040

attgtaagag ccagattcgg ggggtagagt gtggaggtca gagcaactcc accttgaata
5100

agaaggtaaa gcaacctatc ctgaaagcta acctgccatg gtggcttctg attaacctct
5160

gttctaggaa gactgacagt ttgggtctgt gtcattgccc aaatctcatg ttaaattgta
5220

atccccagtg ttcggaggtg ggacttggtg gtaggtgatt cggtcatggg agtagatttt
5280

cttctttgtg gtgttacagt gatagtgagt gagttctcgt gagatctggt catttaaaag
5340

tgtgtggccc ctcccctccc tctcttggtc ctcctactgc catgtaagat acctgctcct
5400

gctttgcctt ctaccataag taaaagcccc ctgaggcctc cccagaagca gatgccacca
5460

tgcttcctgt acagcctgca gaaccatcag ccaattaaac ctcttttctg tataaattac
5520

cagtcttgag tatctcttta cagcagtgtg agaacggact aatacaaggg tctccaaaat
5580

tccaagttta tgtattcttt cttgccaaat agcaggtatt taccataaat cctgtcctta
5640

ggtcaaacaa ccttgatggc atcgtacttc aattgtctta cacattcctt ctgaatgact
5700

cctcccctat ggcatataag ccctgggtct tgggggataa tggcagaggg gtccaccatc
5760

ttgtctggct gccacctgag acacggacat ggcttctgtt ggtaagtctc tattaaatgt
5820

ttctttctaa gaaactggat ttgtcagctt gtttctttgg cctctcagct tcctcagact
5880

ttggggtagg ttgcacaacc ctgcccacca cgaaacaaat gtttaatatg ataaatatgg
5940

atagatataa tccacataaa taaaagctct tggagggccc tcaataattg ttaagagtgt
6000

aaatgtgtcc aaagatggaa aatgtttgag aactactgtc ccagagattt tcctgagttc
6060

tagagtgtgg gaatatagaa cctggagctt ggcttcttca gcctagaatc aggagtatgg
6120

ggctgaagtc tgaagcttgg cttcagcagt ttggggttgg cttccggagc acatatttga
6180

catgttgcga ctgtgatttg gggtttggta tttgctctga atcctaatgt ctgtccttga
6240

ggcatctaga atctgaaatc tgtggtcaga attctattat cttgagtagg acatctccag
6300

tcctggttct gccttctagg gctggagtct gtagtcagtg acccggtctg gcatttcaac
6360

ttcatataca gtgggctatc ttttggtcca tgtttcaacc aaacaaccga ataaaccatt
6420

agaacctttc cccacttccc tagctgcaat gttaaaccta ggatttctgt ttaataggtt
6480

catatgaata atttcagcct gatccaactt tacattcctt ctaccgttat tctacaccca
6540

ccttaaaaat gcattcccaa tatattccct ggattctacc tatatatggt aatcctggct
6600

ttgccagttt ctagtgcatt aacatacctg atttacattc ttttacttta aagtggaaat
6660

aagagtccct ctgcagagtt caggagttct caagatggcc cttacttctg acatcaattg
6720

agatttcaag ggagtcgcca agatcatcct caggttcagt gattgctggt agccctcata
6780

taactcaatg aaagctgtta tgctcatggc tatggtttat tacagcaaaa gaatagagat
6840

gaaaatctag caagggaaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag
6900

attcctgttg ttttctccca gtggtgtcat ggaaagcagt atcttctcca tacaatgatg
6960

tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatattgga
7020

gcttggttgc acagacatgt cgaccacctt catggctgaa ctttagtact tagcccctcc
7080

agacgtctac agctgatagg ctgtaaccca acattgtcac ataaatcac attgttagac
7140

tatccagtgt ggcccaagct cccgtgtaaa cacaggcact ctaaacaggc aggatatttc
7200

aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg
7260

agaatccttt accgcacact ctccttcaca gggttattgt gaggatcaaa tgtggtcatg
7320

tgtgtgagac accagcacat gtctggctgt ggagagtgac ttctatgtgt gctaacattg
7380

ctgagtgcta agaaagtatt aggcatggct ttcagcactc acagatgctc atctaatcct
7440

cacaacatgg ctacagggtg ggcactacta gcctcatttg acagaggaaa ggactgtgga
7500

taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca
7560

tgattagaca ttgtctgcag agaaattatg gctggatgtc tctgccccgg aaaggggat
7620

gcactttcct tgacccccta tctcagatct tgactttgag gttatctcag acttcctcta
7680

tgataccagg agcccatcat aatctctctg tgtcctctcc ccttcctcag tcttactgcc
7740

cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca
7800

gagaactata aatgtgtatc ctacagggga gaaaaaaaaa aagaactctg aaagagctga
7860

cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat
7920

gagactcctg ggtcagaggc aaaagatttt attacccaca gctaaggagg cagcatgaac
7980

tttgtgttca catttgttca ctttgccccc caattcatat gggatgatca gagcagttca
8040

ggtggatgga cacaggggtt tgtggcaaag gtgagcaacc taggcttaga aatcctcaat
8100

cttataagaa ggtactagca aacttgtcca gtctttgtat ctgacggaga tattatcttt
8160

ataattgggt tgaaagcaga cctactctgg aggaacatat tgtatttatt gtcctgaaca
8220

gtaaacaaat ctgctgtaaa atagacgtta actttattat ctaaggcagt aagcaaacct
8280

agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaatatgc ttgaaaagat
8340

ggtccagaaa agaaaacggt attattgcct ttgctcagaa gacacacaga aacataagag
8400

aaccatggaa aattgtctcc caacactgtt cacccagagc cttccactct tgtctgcagg
8460

acagtcttaa catcccatca ttagtgtgtc taccacatct ggcttcaccg tgcctaacca
8520

agatttctag gtccagttcc ccaccatgtt tggcagtgcc ccactgccaa ccccagaata
8580

agggagtgct cagaattccg aggggacatg ggtggggatc agaacttctg ggcttgagtg
8640

cagaggggc ccatactcct tggttccgaa ggaggaagag gctggaggtg aatgtccttg
8700

gaggggagga atgtgggttc tgaactctta aatccccaag ggaggagact ggtaaggtcc
8760

cagcttccga ggtactgacg tgggaatggc ctgagaggtc taagaatccc gtatcctcgg
8820

gaaggagggg ctgaaattgt gaggggttga gttgcagggg tttgttagct tgagactcct
8880

tggtgggtcc ctgggaagca aggactggaa ccattggctc agggtttgg tgtgaaggta
8940

atgggatctc ctgattctca aagggtcaga ggactgagag ttgcccatgc tttgatcttt
9000

ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc
9060

gcctgcgcga gtataatctg cacatgtgcc atgtcccgag gcctggggca tcatccactc
9120

atcattcagc atctgcgcta tgcgggcgag gccggcgcca tgacgtcatg tagctgcgac
9180

tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc
9240

ctggtggatg tggcctgcgt ggtgccaggc cggggcctgg tgtccgataa agatcctaga
9300

accacaggaa accaggactg aaaggtgcta gagaatggcc atatgtcgct gtccatgaaa
9360

tctcaaggac ttctgggtgg agggcacagg agcctgaact tacgggtttg ccccagtcca
9420

ctgtcctccc aagtgagtct cccagatacg aggcactgtg ccagcatcag cttcatctgt
9480

accacatctt gtaacaggga ctacccagga ccctgatgaa caccatggtg tgtgcaggaa
9540

gaggggtga aggcatggac tcctgtgtgg tcagagccca gaggggcca tgacgggtgg
9600

ggaggaggct gtggactggc tcgagaagtg ggatgtggtt gtgtttgatt tcctttggcc
9660

agataaagtg ctggatatag cattgaaaac ggagtatgaa gaccagttag aatggagggt
9720

caggttggag ttgagttaca gatggggtaa aattctgctt cggatgagtt tggggattgg
9780

caatctaaag gtggtttggg atggcatggc tttgggatgg aaataggttt gtttttatgt
9840

tggctgggaa gggtgtgggg attgaattgg ggatgaagta ggtttagttt tggagataga
9900

atacatggag ctggctattg catgcgagga tgtgcattag tttggtttga tctttaaata
9960

aaggaggcta ttagggttgt cttgaattag attaagttgt gttgggttga tgggttgggc
10020

ttgtgggtga tgtggttgga ttgggctgtg ttaaattggt ttgggtcagg ttttggttga
10080

ggttatcatg gggatgagga tatgcttggg acatggattc aggtggttct cattcaagct
10140

gaggcaaatt tcctttcaga cggtcattcc agggaacgag tggttgtgtg ggggaaatca
10200

ggccactggc tgtgaatatc cctctatcct ggtcttgaat tgtgattatc tatgtccatt
10260

ctgtctcctt cactgtactt ggaattgatc tggtcattca gctggaaatg ggggaagatt
10320

ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttcct tctggtttta
10380

ttgaacagat gaaatcacat ttttttttc aaaatcacag aaatcttata gagttaacag
10440

tggactctta taataagagt taacaccagg actcttattc ttgattcttt tctgagacac
10500

caaaatgaga tttctcaatg ccaccctaat tctttttttt ttttttttt tttttgagac
10560

acagtctggg tcttttgctc tgtcactcag gctggagcgc agtggtgtga tcatagctca
10620

ctgaaccctt gacctcctgg acttaaggga tcctcctgct tcagcctcct gagtagatgg
10680

ggctacaggt gcttgccacc acacctggct aattaaattt ttttttttt tttgtagaga
10740

aagggtctca ctttgttgcc ctggctgatc ttgaacttct gacttcaagt gattcttcag
10800

ccttggactc ccaaagcact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca
10860

gcttaatctt ggagtgtata aacctggctc ctgatagcta gacatttcag tgagaaggag
10920

gcattggatt ttgcatgagg acaattctga cctaggaggg caggtcaaca ggaatccccg
10980

ctgtacctgt acgttgtaca ggcatggaga atgaggagtg aggaggccgt accggaaccc
11040

catattgttt agtggacatt ggattttgaa ataataggga acttggtctg ggagagtcat
11100

atttctggat tggacaatat gtggtatcac aaggttttat gatgagggag aaatgtatgt
11160

ggggaaccat tttctgagtg tggaagtgca agaatcagag agtagctgaa tgccaacgct
11220

tctatttcag gaacatggta agttggaggt ccagctctcg ggctcagacg ggtataggga
11280

ccaggaagtc tcacaatccg atcattctga tatttcaggg catattaggt ttggggtgca
11340

aaggaagtac ttgggactta ggcacatgag actttgtatt gaaaatcaat gattggggct
11400

ggccgtggtg ctcacgcctg taatctcatc actttgggag accgaagtgg gaggatggct
11460

tgatctcaag agttggacac cagcctaggc aacatggcca gaccctctct ctacaaaaaa
11520

attaaaaatt agctggatgt ggtggtgcat gcttgtggtc tcagctatcc tggaggctga
11580

gacaggagaa tcggttgagt ctgggagttc aaggctacag ggagctgcga tcacgccgct
11640

gcactccagc ctgggaaaca gagtgagact gtctcagaat ttttttaaaa aagaatcagt
11700

gatcatccca acccctgttg ctgttcatcc tgagcctgcc ttctctggct ttgttcccta
11760

gatcacatct ccatgatcca taggccctgc ccaatctgac ctcacaccgt gggaatgcct
11820

ccagactgat ctagtatgtg tggaacagca agtgctggct ctccctcccc ttccacagct
11880

ctgggtgtgg gaggggttg tccagcctcc agcagcatgg ggagggcctt ggtcagcatc
11940

taggtgccaa cagggcaagg gcggggtcct ggagaatgaa ggctttatag ggctcctcag
12000

ggaggccccc cagccccaaa ctgcaccacc tggccgtgga caccggt
12047

HRE-TRE SEQ ID NO:12

ccccgagg cagtgcat gaggctcagg gcgtgcgt gagtcgcagcgagaccccg gggtgcag
gccgga

PSA-TRE SEQ ID NO:13

aagcttctag ttttcttttc ccggtgacat cgtggaaagc actagcatct ctaagcaatg 60

atctgtgaca atattcacag tgtaatgcca tccagggaac tcaactgagc cttgatgtcc 120

agagattttt gtgtttttt ctgagactga gtctcgctct gtgccaggct ggagtgcagt 180

ggtgcaacct tggctcactg caagctccgc ctcctgggtt cacgccattc tcctgcctca 240

gcctcctgag tagctgggac tacaggcacc cgccaccacg cctggctaat ttttttgtat 300

ttttagtaga gatggggttt cactgtgtta gccaggatgg tctcagtctc ctgacctcgt 360

gatctgccca ccttggcctc ccaaagtgct gggatgacag gcgtgagcca ccgcgcctgg 420

ccgatatcca gagatttttt ggggggctcc atcacacaga catgttgact gtcttcatgg 480

ttgactttta gtatccagcc cctctagaaa tctagctgat atagtgtggc tcaaaacctt 540

cagcacaaat cacaccgtta gactatctgg tgtggcccaa accttcaggt gaacaaaggg 600

actctaatct ggcaggatac tccaaagcat tagagatgac ctcttgcaaa gaaaaagaaa 660

tggaaaagaa aaagaaagaa aggaaaaaaa aaaaaaaaaa gagatgacct ctcaggctct 720

gaggggaaac gcctgaggtc tttgagcaag gtcagtcctc tgttgcacag tctccctcac 780

agggtcattg tgacgatcaa atgtggtcac gtgtatgagg caccagcaca tgcctggctc 840

tggggagtgc cgtgtaagtg tatgcttgca ctgctgaatg gctgggatgt gtcagggatt 900

atcttcagca cttacagatg ctcatctcat cctcacagca tcactatggg atgggtatta 960

ctggcctcat ttgatggaga aagtggctgt ggctcagaaa gggggggacca ctagaccagg 1020

gacactctgg atgctgggga ctccagagac catgaccact caccaactgc agagaaatta
1080

attgtggcct gatgtccctg tcctggagag ggtggaggtg gaccttcact aacctcctac
1140

cttgaccctc tcttttaggg ctctttctga cctccaccat ggtactagga ccccattgta
1200

ttctgtaccc tcttgactct atgaccccca ccgcccactg catccagctg ggtcccctcc
1260

tatctctatt cccagctggc cagtgcagtc tcagtgccca cctgtttgtc agtaactctg
1320

aaggggctga cattttactg acttgcaaac aaataagcta actttccaga gttttgtgaa
1380

tgctggcaga gtccatgaga ctcctgagtc agaggcaaag gcttttactg ctcacagctt
1440

agcagacagc atgaggttca tgttcacatt agtacacctt gccccccccа aatcttgtag
1500

ggtgaccaga gcagtctagg tggatgctgt gcagaagggg tttgtgccac tggtgagaaa
1560

cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagcctttg
1620

tctctgatga agatattatc ttcatgatct tggattgaaa acagacctac tctggaggaa
1680

catattgtat cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat
1740

tatctaggac agtaagcaag cctggatctg agagagatat catcttgcaa ggatgcctgc
1800

tttacaaaca tccttgaaac aacaatccag aaaaaaaaag gtgttactgt ctttgctcag
1860

aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga
1920

gccttccacc ctttctgcag gacagtctca acgttccacc attaaatact tcttctatca
1980

catcccgctt ctttatgcct aaccaaggtt ctaggtcccg atcgactgtg tctggcagca
2040

ctccactgcc aaacccagaa taaggcagcg ctcaggatcc cgaaggggca tggctgggga
2100

tcagaacttc tgggtttgag tgaggagtgg gtccaccctc ttgaatttca aaggaggaag
2160

aggctggatg tgaaggtact gggggaggga aagtgtcagt tccgaactct taggtcaatg
2220

agggaggaga ctggtaaggt cccagctccc gaggtactga tgtgggaatg gcctaagaat
2280

ctcatatcct caggaagaag gtgctggaat cctgaggggt agagttctgg gtatatttgt
2340

ggcttaaggc tctttggccc ctgaaggcag aggctggaac cattaggtcc agggtttggg
2400

gtgatagtaa tgggatctct tgattcctca agagtctgag gatcgagggt tgcccattct
2460

tccatcttgc cacctaatcc ttactccact tgagggtatc accagccctt ctagctccat
2520

gaaggtcccc tgggcaagca caatctgagc atgaaagatg ccccagaggc cttgggtgtc
2580

atccactcat catccagcat cacactctga gggtgtggcc agcaccatga cgtcatgttg
2640

ctgtgactat ccctgcagcg tgcctctcca gccacctgcc aaccgtagag ctgcccatcc
2700

tcctctggtg ggagtggcct gcatggtgcc aggctgaggc ctagtgtcag acagggagcc
2760

tggaatcata gggatccagg actcaaaagt gctagagaat ggccatatgt caccatccat
2820

gaaatctcaa gggcttctgg gtggagggca cagggacctg aacttatggt ttcccaagtc
2880

tattgctctc ccaagtgagt ctcccagata cgaggcactg tgccagcatc agccttatct
2940

ccaccacatc ttgtaaaagg actacccagg gccctgatga acaccatggt gtgtacagga
3000

gtaggggtg gaggcacgga ctcctgtgag gtcacagcca agggagcatc atcatgggtg
3060

gggaggaggc aatggacagg cttgagaacg gggatgtggt tgtatttggt tttctttggt
3120

tagataaagt gctgggtata ggattgagag tggagtatga agaccagtta ggatggagga
3180

tcagattgga gttgggttag ataaagtgct gggtatagga ttgagagtgg agtatgaaga
3240

ccagttagga tggaggatca gattggagtt gggttagaga tggggtaaaa ttgtgctccg
3300

gatgagtttg ggattgacac tgtggaggtg gtttgggatg gcatggcttt gggatggaaa
3360

tagatttgtt ttgatgttgg ctcagacatc cttggggatt gaactgggga tgaagctggg
3420

tttgattttg gaggtagaag acgtggaagt agctgtcaga tttgacagtg gccatgagtt
3480

ttgtttgatg gggaatcaaa caatggggga agacataagg gttggcttgt taggttaagt
3540

tgcgttgggt tgatggggtc ggggctgtgt ataatgcagt tggattggtt tgtattaaat
3600

tgggttgggt caggttttgg ttgaggatga gttgaggata tgcttgggga caccggatcc
3660

atgaggttct cactggagtg gagacaaact tcctttccag gatgaatcca gggaagcctt
3720

aattcacgtg taggggaggt caggccactg gctaagtata tccttccact ccagctctaa
3780

gatggtctta aattgtgatt atctatatcc acttctgtct ccctcactgt gcttggagtt
3840

tacctgatca ctcaactaga aacaggggaa gattttatca aattcttttt ttttttttt
3900

ttttttgag acagagtctc actctgttgc ccaggctgga gtgcagtggc gcagtctcgg
3960

ctcactgcaa cctctgcctc ccaggttcaa gtgattctcc tgcctcagcc tcctgagttg
4020

ctgggattac aggcatgcag caccatgccc agctaatttt tgtattttta gtagagatgg
4080

ggtttcacca atgtttgcca ggctggcctc gaactcctga cctggtgatc cacctgcctc
4140

agcctcccaa agtgctggga ttacaggcgt cagccaccgc gcccagccac ttttgtcaaa
4200

ttcttgagac acagctcggg ctggatcaag tgagctactc tggttttatt gaacagctga
4260

aataaccaac tttttggaaa ttgatgaaat cttacggagt taacagtgga ggtaccaggg
4320

ctcttaagag ttcccgattc tcttctgaga ctacaaattg tgattttgca tgccacctta
4380

atctttttt ttttttttt aaatcgaggt ttcagtctca ttctatttcc caggctggag
4440

ttcaatagcg tgatcacagc tcactgtagc cttgaactcc tggccttaag agattctcct
4500

gcttcggtct cccaatagct aagactacag tagtccacca ccatatccag ataattttta
4560

aatttttgg ggggccgggc acagtggctc acgcctgtaa tcccaacacc atgggaggct
4620

gagatgggtg gatcacgagg tcaggagttt gagaccagcc tgaccaacat ggtgaaactc
4680

tgtctctact aaaaaaaaaa aaaatagaaa aattagccgg gcgtggtggc acacggcacc
4740

tgtaatccca gctactgagg aggctgaggc aggagaatca cttgaaccca gaaggcagag
4800

gttgcaatga gccgagattg cgccactgca ctccagcctg ggtgacagag tgagactctg
4860

tctcaaaaaa aaaaaatttt ttttttttt ttgtagagat ggatcttgct ttgtttctct
4920

ggttggcctt gaactcctgg cttcaagtga tcctcctacc ttggcctcgg aaagtgttgg
4980

gattacaggc gtgagccacc atgactgacc tgtcgttaat cttgaggtac ataaacctgg
5040

ctcctaaagg ctaaaggcta aatatttgtt ggagaagggg cattggattt tgcatgagga
5100

tgattctgac ctgggagggc aggtcagcag gcatctctgt tgcacagata gagtgtacag
5160

gtctggagaa caaggagtgg ggggttattg gaattccaca ttgtttgctg cacgttggat
5220

tttgaaatgc tagggaactt tgggagactc atatttctgg gctagaggat ctgtggacca
5280

caagatcttt ttatgatgac agtagcaatg tatctgtgga gctggattct gggttgggag
5340

tgcaaggaaa agaatgtact aaatgccaag acatctattt caggagcatg aggaataaaa
5400

gttctagttt ctggtctcag agtggtgcat ggatcaggga gtctcacaat ctcctgagtg
5460

ctggtgtctt agggcacact gggtcttgga gtgcaaagga tctaggcacg tgaggctttg
5520

tatgaagaat cggggatcgt acccaccccc tgtttctgtt tcatcctggg catgtctcct
5580

ctgcctttgt cccctagatg aagtctccat gagctacaag ggcctggtgc atccagggtg
5640

atctagtaat tgcagaacag caagtgctag ctctccctcc ccttccacag ctctgggtgt
5700

gggagggggt tgtccagcct ccagcagcat ggggagggcc ttggtcagcc tctgggtgcc
5760

agcagggcag gggcggagtc ctggggaatg aaggttttat agggctcctg ggggaggctc
5820

cccagcccca agctt
5835

CEA TRE SEQ ID NO:14

aagcttttta gtgctttaga cagtgagctg gtctgtctaa cccaagtgac ctgggctcca 60

tactcagccc cagaagtgaa gggtgaagct gggtggagcc aaaccaggca agcctaccct 120

cagggctccc agtggcctga gaaccattgg acccaggacc cattacttct agggtaagga 180

aggtacaaac accagatcca accatggtct ggggggacag ctgtcaaatg cctaaaaata 240

tacctgggag aggagcaggc aaactatcac tgccccaggt tctctgaaca gaaacagagg 300

ggcaacccaa agtccaaatc caggtgagca ggtgcaccaa atgcccagag atatgacgag 360

gcaagaagtg aaggaaccac ccctgcatca aatgttttgc atgggaagga gaagggggtt 420

gctcatgttc ccaatccagg agaatgcatt tgggatctgc cttcttctca ctccttggtt 480

agcaagacta agcaaccagg actctggatt tggggaaaga cgtttatttg tggaggccag 540

tgatgacaat cccacgaggg cctaggtgaa gagggcagga aggctcgaga cactggggac 600

tgagtgaaaa ccacacccat gatctgcacc acccatggat gctccttcat tgctcacctt 660

tctgttgata tcagatggcc ccatttctg taccttcaca gaaggacaca ggctagggtc 720

tgtgcatggc cttcatcccc ggggccatgt gaggacagca ggtgggaaag atcatgggtc 780

ctcctgggtc ctgcagggcc agaacattca tcacccatac tgacctccta gatgggaatg 840

gcttccctgg ggctgggcca acggggcctg ggcaggggag aaaggacgtc aggggacagg 900

gaggaagggt catcgagacc cagcctggaa ggttcttgtc tctgaccatc caggatttac 960

ttccctgcat ctacctttgg tcattttccc tcagcaatga ccagctctgc ttcctgatct 1020

cagcctccca ccctggacac agcaccccag tccctggccc ggctgcatcc acccaatacc 1080

ctgataaccc aggacccatt acttctaggg taaggagggt ccaggagaca gaagctgagg 1140

aaaggtctga agaagtcaca tctgtcctgg ccagagggga aaaaccatca gatgctgaac 1200

caggagaatg ttgacccagg aaagggaccg aggacccaag aaaggagtca gaccaccagg 1260

gtttgcctga gaggaaggat caaggccccg agggaaagca gggctggctg catgtgcagg 1320

acactggtgg ggcatatgtg tcttagattc tccctgaatt cagtgtccct gccatggcca 1380

gactctctac tcaggcctgg acatgctgaa ataggacaat ggccttgtcc tctctcccca 1440

ccatttggca agagacataa aggacattcc aggacatgcc ttcctgggag gtccaggttc 1500

tctgtctcac acctcaggga ctgtagttac tgcatcagcc atggtaggtg ctgatctcac 1560

ccagcctgtc caggcccttc cactctccac tttgtgacca tgtccaggac caccctcag 1620

atcctgagcc tgcaaatacc cccttgctgg gtgggtggat tcagtaaaca gtgagctcct 1680

atccagcccc cagagccacc tctgtcacct tcctgctggg catcatccca ccttcacaag 1740

cactaaagag catggggaga cctggctagc tgggtttctg catcacaaag aaaataatcc 1800

cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccaggagata 1860

gcagaggtca gccctaggga gggtgggtca tccacccagg ggacaggggt gcaccagcct 1920

tgctactgaa agggcctccc caggacagcg ccatcagccc tgcctgagag ctttgctaaa 1980

cagcagtcag aggaggccat ggcagtggct gagctcctgc tccaggcccc aacagaccag 2040

accaacagca caatgcagtc cttccccaac gtcacaggtc accaaaggga aactgaggtg 2100

ctacctaacc ttagagccat caggggagat aacagcccaa tttcccaaac aggccagttt 2160

caatcccatg acaatgacct ctctgctctc attcttccca aaataggacg ctgattctcc 2220

cccaccatgg atttctccct tgtcccggga gccttttctg ccccctatga tctgggcact 2280

cctgacacac acctcctctc tggtgacata tcagggtccc tcactgtcaa gcagtccaga 2340

aaggacagaa ccttggacag cgcccatctc agcttcaccc ttcctccttc acagggttca 2400

gggcaaagaa taaatggcag aggccagtga gcccagagat ggtgacaggc agtgacccag 2460

gggcagatgc ctggagcagg agctggcggg gccacaggga gaaggtgatg caggaaggga 2520

aacccagaaa tgggcaggaa aggaggacac aggctctgtg gggctgcagc ccagggttgg 2580

actatgagtg tgaagccatc tcagcaagta aggccaggtc ccatgaacaa gagtgggagc 2640

acgtggcttc ctgctctgta tatggggtgg gggattccat gccccataga accagatggc 2700

cggcgttcag atggagaagg agcaggacag gggatcccca ggataggagg acacccagtgt 2760

ccccacccag gcaggtgact gatgaatggg catgcagggt cctcctgggc tgggctctcc 2820

ctttgtccct caggattcct tgaaggaaca tccggaagcc gaccacatct acctggtggg 2880

ttctggggag tccatgtaaa gccaggagct tgtgttgcta ggaggggtca tggcatgtgc 2940

tgggggcacc aaagagagaa acctgagggc aggcaggacc tggtctgagg aggcatggga 3000

gcccagatgg ggagatggat gtcaggaaag gctgccccat cagggagggt gatagcaatg 3060

gggggtctgt gggagtgggc acgtgggatt ccctgggctc tgccaagttc cctcccatag 3120

tcacaacctg gggacactgc ccatgaaggg gcgcctttgc ccagccagat gctgctggtt 3180

ctgcccatcc actaccctct ctgctccagc cactctgggt ctttctccag atgccctgga 3240

cagccctggc ctgggcctgt cccctgagag gtgttgggag aagctgagtc tctggggaca 3300

ctctcatcag agtctgaaag gcacatcagg aaacatccct ggtctccagg actaggcaat 3360

gaggaaaggg ccccagctcc tccctttgcc actgagaggg tcgaccctgg gtggccacag 3420

tgacttctgc gtctgtccca gtcaccctga aaccacaaca aaaccccagc cccagaccct 3480

gcaggtacaa tacatgtggg gacagtctgt acccagggga agccagttct ctcttcctag 3540

gagaccgggc ctcagggctg tgccccgggc aggcgggggc agcacgtgcc tgtccttgag 3600

aactcgggac cttaagggtc tctgctctgt gaggcacagc aaggatcctt ctgtccagag 3660

atgaaagcag ctcctgcccc tcctctgacc tcttcctcct tcccaaatct caaccaacaa 3720

ataggtgttt caaatctcat catcaaatct tcatccatcc acatgagaaa gcttaaaacc 3780

caatggattg acaacatcaa gagttggaac aagtggacat ggagatgtta cttgtggaaa 3840

tttagatgtg ttcagctatc gggcaggaga atctgtgtca aattccagca tggttcagaa 3900

gaatcaaaaa gtgtcacagt ccaaatgtgc aacagtgcag gggataaaac tgtggtgcat 3960

tcaaactgag ggatattttg gaacatgaga aaggaaggga ttgctgctgc acagaacatg 4020

gatgatctca cacatagagt tgaaagaaag gagtcaatcg cagaatagaa aatgatcact 4080

aattccacct ctataaagtt tccaagagga aaacccaatt ctgctgctag agatcagaat 4140

ggaggtgacc tgtgccttgc aatggctgtg agggtcacgg gagtgtcact tagtgcaggc 4200

aatgtgccgt atcttaatct gggcaggcct ttcatgagca cataggaatg cagacattac 4260

tgctgtgttc attttacttc accggaaaag aagaataaaa tcagccgggc gcggtggctc 4320

acgcctgtaa tcccagcact ttagaaggct gaggtgggca gattacttga ggtcaggagt 4380

tcaagaccac cctggccaat atggtgaaac cccggctcta ctaaaaatac aaaaattagc 4440

tgggcatggt ggtgcgcgcc tgtaatccca gctactcggg aggctgaggc tggacaattg 4500

cttggaccca ggaagcagag gttgcagtga gccaagattg tgccactgca ctccagcttg 4560

ggcaacagag ccagactctg taaaaaaaaa aaaaaaaaaa aaaaaaagaa agaaagaaaa 4620

agaaaagaaa gtataaaatc tctttgggtt aacaaaaaaa gatccacaaa acaaacacca 4680

gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc 4740

acttttgtgg caataaaacc ttcatgtcaa aaggagacca ggacacaatg aggaagtaaa 4800

actgcaggcc ctacttgggt gcagagaggg aaaatccaca aataaaacat taccagaagg 4860

agctaagatt tactgcattg agttcattcc ccaggtatgc aaggtgattt taacacctga 4920

aaatcaatca ttgcctttac tacatagaca gattagctag aaaaaaatta caactagcag 4980

aacagaagca atttggcctt cctaaaattc cacatcatat catcatgatg gagacagtgc 5040

agacgccaat gacaataaaa agagggacct ccgtcacccg gtaaacatgt ccacacagct 5100

ccagcaagca eccgtcttcc cagtgaatca ctctaacctc ccctttaatc agccccaggc 5160

aaggctgcct gcgatggcca cacaggctcc aacccgtggg cctcaacctc ccgcagaggc 5220

tctcctttgg ccaccccatg gggagagcat gaggacaggg cagagccctc tgatgcccac 5280

acatggcagg agctgacgcc agagccatgg gggctggaga gcagagctgc tggggtcaga 5340

gcttcctgag gacacccagg cctaagggaa ggcagctccc tggatggggg caaccaggct 5400

ccgggctcca acctcagagc ccgcatggga ggagccagca ctctaggcct ttcctagggt 5460

gactctgagg ggaccctgac acgacaggat cgctgaatgc acccgagatg aaggggccac 5520

cacgggaccc tgctctcgtg gcagatcagg agagagtggg acaccatgcc aggcccccat 5580

ggcatggctg cgactgaccc aggccactcc cctgcatgca tcagcctcgg taagtcacat 5640

gaccaagccc aggaccaatg tggaaggaag gaaacagcat ccccttttagt gatggaaccc 5700

aaggtcagtg caaagagagg ccatgagcag ttaggaaggg tggtccaacc tacagcacaa 5760

accatcatct atcataagta gaagccctgc tccatgaccc ctgcatttaa ataaacgttt 5820

gttaaatgag tcaaattccc tcaccatgag agctcacctg tgtgtaggcc catcacacac 5880

acaaacacac acacacacac acacacacac acacacacac acagggaaag tgcaggatcc 5940

tggacagcac caggcaggct tcacaggcag agcaaacagc gtgaatgacc catgcagtgc 6000

cctgggcccc atcagctcag agaccctgtg agggctgaga tggggctagg caggggagag 6060

acttagagag ggtggggcct ccagggaggg ggctgcaggg agctgggtac tgccctccag 6120

ggaggggget gcagggagct gggtactgcc ctccagggag ggggctgcag ggagctgggt 6180

actgccctcc agggaggggg ctgcagggag ctgggtactg ccctccaggg agggggctgc 6240

agggagctgg gtactgccct ccagggaggc aggagcactg ttcccaacag agagcacatc 6300

ttcctgcagc agctgcacag acacaggagc ccccatgact gccctgggcc agggtgtgga 6360

ttccaaattt cgtgccccat tgggtgggac ggaggttgac cgtgacatcc aaggggcatc 6420

tgtgattcca aacttaaact actgtgccta caaaatagga aataacccta ctttttctac 6480

tatctcaaat tccctaagca caagctagca ccctttaaat caggaagttc agtcactcct 6540

ggggtcctcc catgccccca gtctgacttg caggtgcaca gggtggctga catctgtcct 6600

tgctcctcct cttggctcaa ctgccgcccc tcctgggggt gactgatggt caggacaagg 6660

gatcctagag ctggccccat gattgacagg aaggcaggac ttggcctcca ttctgaagac 6720

taggggtgtc aagagagctg ggcatcccac agagctgcac aagatgacgc ggacagaggg 6780

tgacacaggg ctcagggctt cagacgggtc gggaggctca gctgagagtt cagggacaga 6840

cctgaggagc ctcagtggga aaagaagcac tgaagtggga agttctggaa tgttctggac 6900

aagcctgagt gctctaagga aatgctccca ccccgatgta gcctgcagca ctggacggtc 6960

tgtgtacctc cccgctgccc atcctctcac agcccccgcc tctagggaca caactcctgc 7020

cctaacatgc atctttcctg tctcattcca cacaaaaggg cctctggggt ccctgttctg 7080

cattgcaagg agtggaggtc acgttcccac agaccaccca gcaacagggt cctatggagg 7140

tgcggtcagg aggatcacac gtcccccccat gcccagggga ctgactctgg gggtgatgga 7200

ttggcctgga ggccactggt cccctctgtc cctgagggga atctgcaccc tggaggctgc 7260

cacatccctc ctgattcttt cagctgaggg cccttcttga aatcccaggg aggactcaac 7320

ccccactggg aaaggcccag tgtggacggt tccacagcag cccagctaag gcccttggac 7380

acagatcctg agtgagagaa cctttaggga cacaggtgca cggccatgtc ccctgtgccc 7440

acacagagca ggggcatctg gaccctgagt gtgtagctcc cgcgactgaa cccagccctt 7500

ccccaatgac gtgacccctg gggtggctcc aggtctccag tccatgccac caaaatctcc 7560

agattgaggg tcctcccttg agtccctgat gcctgtccag gagctgcccc ctgagcaaat 7620

ctagagtgca gagggctggg attgtggcag taaaagcagc cacatttgtc tcaggaagga 7680

aagggaggac atgagctcca ggaagggcga tggcgtcctc tagtgggcgc ctcctgttaa 7740

tgagcaaaaa ggggccagga gagttgagag atcagggctg gccttggact aaggctcaga 7800

tggagaggac tgaggtgcaa agaggggggct gaagtagggg agtggtcggg agagatggga 7860

ggagcaggta aggggaagcc ccagggaggc cgggggaggg tacagcagag ctctccactc 7920

ctcagcattg acatttgggg tggtcgtgct agtggggttc tgtaagttgt agggtgttca 7980

gcaccatctg gggactctac ccactaaatg ccagcaggac tccctcccca agctctaaca 8040

accaacaatg tctccagact ttccaaatgt cccctggaga gcaaaattgc ttctggcaga 8100

atcactgatc tacgtcagtc tctaaaagtg actcatcagc gaaatccttc acctcttggg 8160

agaagaatca caagtgtgag aggggtagaa actgcagact tcaaaatctt tccaaaagag 8220

ttttacttaa tcagcagttt gatgtcccag gagaagatac atttagagtg tttagagttg 8280

atgccacatg gctgcctgta cctcacagca ggagcagagt gggttttcca agggcctgta 8340

accacaactg gaatgacact cactgggtta cattacaaag tggaatgtgg ggaattctgt 8400

agactttggg aagggaaatg tatgacgtga gcccacagcc taaggcagtg gacagtccac 8460

tttgaggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta 8520

gaaaacatgt-tttattaaga ggaaaaatct aggctagaag tgcttcatgc tctttttttct 8580

ctttatgttc aaattcatat acttttagat cattccttaa agaagaatct atccccctaa 8640

gtaaatgtta tcactgactg gatagtgttg gtgtctcact cccaacccct gtgtggtgac 8700

agtgccctgc ttccccagcc ctgggccctc tctgattcct gagagctttg ggtgctcctt 8760

cattaggagg aagagaggaa gggtgttttt aatattctca ccattcaccc atccacctct 8820

tagacactgg gaagaatcag ttgcccactc ttggatttga tcctcgaatt aatgacctct 8880

atttctgtcc cttgtccatt tcaacaatgt gacaggccta agaggtgcct tctccatgtg 8940

atttttgagg agaaggttct caagataagt tttctcacac ctctttgaat tacctccacc 9000

tgtgtcccca tcaccattac cagcagcatt tggacccttt ttctgttagt cagatgcttt 9060

ccacctcttg agggtgtata ctgtatgctc tctacacagg aatatgcaga ggaaatagaa 9120

aaagggaaat cgcattacta ttcagagaga agaagacctt tatgtgaatg aatgagagtc 9180

taaaatccta agagagccca tataaaatta ttaccagtgc taaaactaca aaagttacac 9240

taacagtaaa ctagaataat aaaacatgca tcacagttgc tggtaaagct aaatcagata 9300

ttttttttct agaaaaagca ttccatgtgt gttgcagtga tgacaggagt gcccttcagt 9360

caatatgctg cctgtaattt ttgttccctg gcagaatgta ttgtcttttc tccctttaaa 9420

tcttaaatgc aaaactaaag gcagctcctg ggcccccctcc ccaaagtcag ctgcctgcaa 9480

ccagccccac gaagagcaga ggcctgagct tccctggtca aaataggggg ctagggagct 9540

taaccttgct cgataaagct gtgttcccag aatgtcgctc ctgttcccag gggcaccagc 9600

ctggagggtg gtgagcctca ctggtggcct gatgcttacc ttgtgccctc acaccagtgg 9660

tcactggaac cttgaacact tggctgtcgc ccggatctgc agatgtcaag aacttctgga 9720

agtcaaatta ctgcccactt ctccagggca gatacctgtg aacatccaaa accatgccac 9780

agaaccctgc ctggggtcta caacacatat ggactgtgag caccaagtcc agccctgaat 9840

ctgtgaccac ctgccaagat gcccctaact gggatccacc aatcactgca catggcaggc 9900

agcgaggctt ggaggtgctt cgccacaagg cagccccaat ttgctgggag tttcttggca 9960

cctggtagtg gtgaggagcc ttgggaccct caggattact ccccttaagc atagtgggga 10020

cccttctgca tccccagcag gtgccccgct cttcagagcc tctctctctg aggtttaccc 10080

agacccctgc accaatgaga ccatgctgaa gcctcagaga gagagatgga gctttgacca 10140

ggagccgctc ttccttgagg gccagggcag ggaaagcagg aggcagcacc aggagtggga 10200

acaccagtgt ctaagcccct gatgagaaca gggtggtctc tcccatatgc ccataccagg 10260

cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgtttcccag 10320

cctaacgtgc agccatgccc atctacccac tgcctactgc aggacagcac caacccagga 10380

gctgggaagc tgggagaaga catggaatac ccatggcttc tcaccttcct ccagtccagt 10440

gggcaccatt tatgcctagg acacccacct gccggcccca ggctcttaag agttaggtca 10500

cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aacccgggag gcagaggttg 10560

cagtgagccg agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc 10620

aaaaaaaaag agaaagatag catcagtggc taccaagggc taggggcagg ggaaggtgga 10680

gagttaatga ttaatagtat gaagtttcta tgtgagatga tgaaaatgtt ctggaaaaaa 10740

aaatatagtg gtgaggatgt agaatattgt gaatataatt aacggcattt aattgtacac 10800

ttaacatgat taatgtggca tattttatct tatgtatttg actacatcca agaaacactg 10860

ggagagggaa agcccaccat gtaaaataca cccaccctaa tcagatagtc ctcattgtac 10920

ccaggtacag gcccctcatg acctgcacag gaataactaa ggatttaagg acatgaggct 10980

tcccagccaa ctgcaggtgc acaacataaa tgtatctgca aacagactga gagtaaagct 11040

gggggcacaa acctcagcac tgccaggaca cacacccttc tcgtggattc tgactttatc 11100

tgacccggcc cactgtccag atcttgttgt gggattggga caagggaggt cataaagcct 11160

gtccccaggg cactctgtgt gagcacacga gacctccccа ccccccccacc gttaggtctc 11220

cacacataga tctgaccatt aggcattgtg aggaggactc tagcgcgggc tcagggatca 11280

caccagagaa tcaggtacag agaggaagac ggggctcgag gagctgatgg atgacacaga 11340

gcagggttcc tgcagtccac aggtccagct caccctggtg taggtgcccc atccccctga 11400

tccaggcatc cctgacacag ctcccctccg gagcctcctc ccaggtgaca catcagggtc 11460

cctcactcaa gctgtccaga gagggcagca ccttggacag cgcccacccc acttcactct 11520

tcctccctca cagggctcag ggctcagggc tcaagtctca gaacaaatgg cagaggccag 11580

tgagcccaga gatggtgaca gggcaatgat ccaggggcag ctgcctgaaa cgggagcagg 11640

tgaagccaca gatgggagaa gatggttcag gaagaaaaat ccaggaatgg gcaggagagg 11700

agaggaggac acaggctctg tggggctgca gcccaggatg ggactaagtg tgaagacatc 11760

tcagcaggtg aggccaggtc ccatgaacag agaagcagct cccacctccc ctgatgcacg 11820

gacacacaga gtgtgtggtg ctgtgccccc agagtcgggc tctcctgttc tggtccccag 11880

ggagtgagaa gtgaggttga cttgtccctg ctcctctctg ctaccccaac attcaccttc 11940

tcctcatgcc cctctctctc aaatatgatt tggatctatg tccccgccca aatctcatgt 12000

caaattgtaa accccaatgt tggaggtggg gccttgtgag aagtgattgg ataatgcggg 12060

tggattttct gctttgatgc tgtttctgtg atagagatct cacatgatct ggttgtttaa 12120

aagtgtgtag cacctctccc ctctctctct ctctctctta ctcatgctct gccatgtaag 12180

acgttcctgt ttcccccttca ccgtccagaa tgattgtaag ttttctgagg cctccccagg 12240

agcagaagcc actatgcttc ctgtacaact gcagaatgat gagcgaatta aacctctttt 12300

ctttataaat taccccagtct caggtatttc tttatagcaa tgcgaggaca gactaataca 12360

atcttctact cccagatccc cgcacacgct tagccccaga catcactgcc cctgggagca 12420

tgcacagcgc agcctcctgc cgacaaaagc aaagtcacaa aaggtgacaa aaatctgcat 12480

ttggggacat ctgattgtga aagagggagg acagtacact tgtagccaca gagactgggg 12540

ctcaccgagc tgaaacctgg tagcactttg gcataacatg tgcatgaccc gtgttcaatg 12600

tctagagatc agtgttgagt aaaacagcct ggtctggggc cgctgctgtc cccacttccc 12660

tcctgtccac cagagggcgg cagagttcct cccaccctgg agcctcccca ggggctgctg 12720

acctccctca gccgggccca cagcccagca gggtccaccc tcacccgggt cacctcggcc 12780

cacgtcctcc tcgccctccg agctcctcac acggactctg tcagctcctc cctgcagcct 12840

atcggccgcc cacctgaggc ttgtcggccg cccacttgag gcctgtcggc tgccctctgc 12900

aggcagctcc tgtcccctac accccctcct tccccgggct cagctgaaag ggcgtctccc 12960

agggcagctc cctgtgatct ccaggacagc tcagtctctc acaggctccg acgcccccta 13020

tgctgtcacc tcacagccct gtcattacca ttaactcctc agtcccatga agttcactga 13080

gcgcctgtct cccggttaca ggaaaactct gtgacaggga ccacgtctgt cctgctctct 13140

gtggaatccc agggcccagc ccagtgcctg acacggaaca gatgctccat aaatactggt 13200

taaatgtgtg ggagatctct aaaaagaagc atatcacctc cgtgtggccc ccagcagtca 13260

gagtctgttc catgtggaca caggggcact ggcaccagca tgggaggagg ccagcaagtg 13320

cccgcggctg ccccaggaat gaggcctcaa cccccagagc ttcagaaggg aggacagagg 13380

cctgcaggga atagatcctc cggcctgacc ctgcagccta atccagagtt cagggtcagc 13440

tcacaccacg tcgaccctgg tcagcatccc tagggcagtt ccagacaagg ccggaggtct 13500

cctcttgccc tccaggggct gacattgcac acagacatca ctcaggaaac ggattccccc 13560

ggacacgaac ctggctttgc taaggaagtg gaggtggagc ctggtttcca tcccttgctc 13620

caacagaccc ttctgatctc tcccacatac ctgctctgtt cctttctcgg tcctatgagg 13680

accctgttct gccaggggtc cctgtgcaac tccagactcc ctcctggtac caccatgggg 13740

aaggtggggt gatcacagga cagtcagcct cgcagagaca gagaccaccc aggactgtca 13800

gggagaacat ggacaggccc tgagccgcag ctcagccaac agacacggag agggagggtc 13860

cccctggagc cttccccaag gacagcagag cccagagtca cccacctccc tccaccacag 13920

tcctctcttt ccaggacaca caagacacct cccctccac atgcaggatc tggggactcc 13980

tgagacctct gggcctgggt ctccatccct gggtcagtgg cggggttggt ggtactggag 14040

acagagggct ggtccctccc cagccaccac ccagtgagcc ttttttctagc ccccagagcc 14100

acctctgtca ccttcctgtt gggcatcatc ccaccttccc agagccctgg agagcatggg 14160

gagacccggg accctgctgg gtttctctgt cacaaaggaa aataatcccc ctggtgtgac 14220

agacccaagg acagaacaca gcagaggtca gcactgggga agacaggttg tcctcccagg 14280

ggatgggggt ccatccacct tgccgaaaag atttgtctga ggaactgaaa atagaaggga 14340

aaaaagagga gggacaaaag aggcagaaat gagaggggag cggacagagg acacctgaat 14400

aaagaccaca cccatgaccc acgtgatgct gagaagtact cctgccctag gaagagactc 14460

transcription start site

agggcagagg gaggaaggac agcagaccag acagtcacag cagccttgac aaaacgttcc 14520

tggaactcaa gctcttctcc acagaggagg acagagcaga cagcagagac catggagtct 14580

ccctcggccc ctccccacag atggtgcatc ccctggcaga ggctcctgct cacaggtgaa 14640

gggaggacaa cctgggagag ggtgggagga gggagctggg gtctcctgcg taggacaggg 14700

ctgtgagacg gacagagggc tcctgttgga gcctgaatag ggaagaggac atcagagagg 14760

gacaggagtc acaccagaaa aatcaaattg aactggaatt ggaaagggcc aggaaaacct 14820

caagagttct attttcctag ttaattgtca ctggccacta cgtttttaaa aatcataata 14880

actgcatcag atgacacttt aaataaaaac ataaccaggg catgaaacac tgtcctcatc 14940

cgcctaccgc ggacattgga aaataagccc caggctgtgg agggccctgg gaaccctcat 15000

gaactcatcc acaggaatct gcagcctgtc ccaggcactg gggtgcaacc aagatc 15056

Mucin-TRE SEQ ID NO: 15

cgagcggccc ctcagcttcg gcgcccagcc ccgcaaggct cccggtgacc actagagggc 60

gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcatcggag 120

cccaggttta ctcccttaag tggaaatttc ttcccccact cctccttggc tttctccaag 180

gagggaaccc aggctgctgg aaagtccggc tggggcgggg actgtgggtt caggggagaa 240

cggggtgtgg aacgggacag ggagcggtta gaagggtggg gctattccgg gaagtggtgg 300

ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctcgg 360

ccgctctgct tcagtggacc cggggagggc ggggaagtgg agtgggagac ctaggggtgg 420

gcttcccgac cttgctgtac aggacctcga cctagctggc tttgttcccc atccccacgt 480

tagttgttgc cctgaggcta aaactagagc ccaggggccc caagttccag actgcccctc 540

ccccctcccc cggagccagg gagtggttgg tgaaaggggg aggccagctg gagaacaaac 600

gggtagtcag ggggttgagc gattagagcc cttgtaccct acccaggaat ggttggggag 660

gaggaggaag aggtaggagg taggggaggg ggcggggttt tgtcacctgt cacctgctcg 720

ctgtgcctag ggcgggcggg cggggagtgg ggggaccggt ataaagcggt aggcgcctgt 780

gcccgctcca cctctcaagc agccagcgcc tgcctgaatc tgttctgccc cctccccacc 840

catttcacca ccaccatg 858

αFP-TRE SEQ ID NO:16

gaattcttag aaatatgggg gtaggggtgg tggtggtaat tctgttttca ccccataggt 60

gagataagca ttgggttaaa tgtgctttca cacacacatc acatttcata agaattaagg 120

aacagactat gggctggagg actttgagga tgtctgtctc ataacacttg ggttgtatct 180

gttctatggg gcttgtttta agcttggcaa cttgcaacag ggttcactga ctttctcccc 240

aagcccaagg tactgtcctc ttttcatatc tgttttgggg cctctggggc ttgaatatct 300

gagaaaatat aaacatttca ataatgttct gtggtgagat gagtatgaga gatgtgtcat 360

tcatttgtat caatgaatga atgaggacaa ttagtgtata aatccttagt acaacaatct 420

gagggtaggg gtggtactat tcaatttcta tttataaaga tacttatttc tatttattta 480

tgcttgtgac aaatgttttg ttcgggacca caggaatcac aaagatgagt ctttgaattt 540

aagaagttaa tggtccagga ataattacat agcttacaaa tgactatgat ataccatcaa 600

acaagaggtt ccatgagaaa ataatctgaa aggtttaata agttgtcaaa ggtgagaggg 660

ctcttctcta gctagagact aatcagaaat acattcaggg ataattattt gaatagacct 720

taagggttgg gtacattttg ttcaagcatt gatggagaag gagagtgaat atttgaaaac 780

attttcaact aaccaaccac ccaatccaac aaacaaaaaa tgaaaagaat ctcagaaaca 840

gtgagataag agaaggaatt ttctcacaac ccacacgtat agctcaactg ctctgaagaa 900

gtatatatct aatatttaac actaacatca tgctaataat gataataatt actgtcattt 960

tttaatgtct ataagtacca ggcatttaga agatattatt ccatttatat atcaaaataa
1020

acttgagggg atagatcatt ttcatgatat atgagaaaaa ttaaaaacag attgaattat
1080

ttgcctgtca tacagctaat aattgaccat aagacaatta gatttaaatt agttttgaat
1140

ctttctaata ccaaagttca gtttactgtt ccatgttgct tctgagtggc ttcacagact
1200

tatgaaaaag taaacggaat cagaattaca tcaatgcaaa agcattgctg tgaactctgt
1260

acttaggact aaactttgag caataacaca catagattga ggattgtttg ctgttagcat
1320

acaaactctg gttcaaagct cctctttatt gcttgtcttg gaaaatttgc tgttcttcat
1380

ggtttctctt ttcactgcta tctatttttc tcaaccactc acatggctac aataactgtc
1440

tgcaagctta tgattcccaa atatctatct ctagcctcaa tcttgttcca gaagataaaa
1500

agtagtattc aaatgcacat caacgtctcc acttggaggg cttaaagacg tttcaacata
1560

caaaccgggg agttttgcct ggaatgtttc ctaaaatgtg tcctgtagca catagggtcc
1620

tcttgttcct taaaatctaa ttacttttag cccagtgctc atcccaccta tggggagatg
1680

agagtgaaaa gggagcctga ttaataatta cactaagtca ataggcatag agccaggact
1740

gtttgggtaa actggtcact ttatcttaaa ctaaatatat ccaaaactga acatgtactt
1800

agttactaag tctttgactt tatctcattc ataccactca gctttatcca ggccacttat
1860

ttgacagtat tattgcgaaa acttcctaac tggtctcctt atcatagtct tatccccttt
1920

tgaaacaaaa gagacagttt caaaatacaa atatgatttt tattagctcc cttttgttgt
1980

ctataatagt cccagaagga gttataaact ccatttaaaa agtctttgag atgtggccct
2040

tgccaacttt gccaggaatt cccaatatct agtattttct actattaaac tttgtgcctc
2100

ttcaaaactg cattttctct cattccctaa gtgtgcattg ttttccctta ccggttggtt
2160

tttccaccac cttttacatt ttcctggaac actatacccct ccctcttcat ttggcccacc
2220

tctaattttc tttcagatct ccatgaagat gttacttcct ccaggaagcc ttatctgacc
2280

cctccaaaga tgtcatgagt tcctcttttc attctactaa tcacagcatc catcacacca
2340

tgttgtgatt actgatacta ttgtctgttt ctctgattag gcagtaagct caacaagagc
2400

tacatggtgc ctgtctcttg ttgctgatta ttcccatcca aaaacagtgc ctggaatgca
2460

gacttaacat tttattgaat gaataaataa aaccccatct atcgagtgct actttgtgca
2520

agacccggtt ctgaggcatt tatatttatt gatttattta attctcattt aaccatgaag
2580

gaggtactat cactatcctt attttatagt tgataaagat aaagcccaga gaaatgaatt
2640

aactcaccca aagtcatgta gctaagtgac agggcaaaaa ttcaaaccag ttccccaact
2700

ttacgtgatt aatactgtgc tatactgcct ctctgatcat atggcatgga atgcagacat
2760

ctgctccgta aggcagaata tggaaggaga ttggaggatg acacaaaacc agcataatat
2820

cagaggaaaa gtccaaacag gacctgaact gatagaaaag ttgttactcc tggtgtagtc
2880

gcatcgacat cttgatgaac tggtggctga cacaacatac attggcttga tgtgtacata
2940

ttatttgtag ttgtgtgtgt atttttatat atatatttgt aatattgaaa tagtcataat
3000

ttactaaagg cctaccattt gccaggcatt tttacatttg tcccctctaa tcttttgatg
3060

agatgatcag attggattac ttggccttga agatgatata tctacatcta tatctatatc
3120

tatatctata tctatatcta tatctatatc tatatctata tatgtatatc agaaaagctg
3180

aaatatgttt tgtaaagtta taaagatttc agactttata gaatctggga tttgccaaat
3240

gtaacccctt tctctacatt aaacccatgt tggaacaaat acatttatta ttcattcatc
3300

aaatgttgct gagtcctggc tatgaaccag acactgtgaa agcctttggg atattttgcc
3360

catgcttggg caagcttata tagtttgctt cataaaactc tatttcagtt cttcataact
3420

aatacttcat gactattgct tttcaggtat tccttcataa caaatacttt ggctttcata
3480

tatttgagta aagtcccccc tgaggaagag tagaagaact gcactttgta aatactatcc
3540

tggaatccaa acggatagac aaggatggtg ctacctcttt ctggagagta cgtgagcaag
3600

gcctgttttg ttaacatgtt ccttaggaga caaaacttag gagagacacg catagcagaa
3660

aatggacaaa aactaacaaa tgaatgggaa ttgtacttga ttagcattga agaccttgtt
3720

tatactatga taaatgtttg tatttgctgg aagtgctact gacggtaaac ccttttttgtt
3780

taaatgtgtg ccctagtagc ttgcagtatg atctattttt taagtactgt acttagctta
3840

tttaaaaatt ttatgtttaa aattgcatag tgctctttca ttgaagaagt tttgagagag
3900

agatagaatt aaattcactt atcttaccat ctagagaaac ccaatgttaa aactttgttg
3960

tccattattt ctgtctttta ttcaacattt tttttagagg gtgggaggaa tacagaggag
4020

gtacaatgat acacaaatga gagcactctc catgtattgt tttgtcctgt ttttcagtta
4080

acaatatatt atgagcatat ttccatttca ttaaatattc ttccacaaag ttattttgat
4140

ggctgtatat caccctactt tatgaatgta ccatattaat ttatttcctg gtgtgggtta
4200

tttgatttta taatcttacc tttagaataa tgaaacacct gtgaagcttt agaaaatact
4260

ggtgcctggg tctcaactcc acagattctg atttaactgg tctgggttac agactaggca
4320

ttgggaattc aaaaagttcc cccagtgatt ctaatgtgta gccaagatcg ggaacccttg
4380

tagacaggga tgataggagg tgagccactc ttagcatcca tcatttagta ttaacatcat
4440

catcttgagt tgctaagtga atgatgcacc tgacccactt tataaagaca catgtgcaaa
4500

taaaattatt ataggacttg gtttattagg gcttgtgctc taagttttct atgttaagcc
4560

atacatcgca tactaaatac tttaaaatgt accttattga catacatatt aagtgaaaag
4620

tgtttctgag ctaaacaatg acagcataat tatcaagcaa tgataatttg aaatgaattt
4680

attattctgc aacttaggga caagtcatct ctctgaattt tttgtacttt gagagtattt
4740

gttatatttg caagatgaag agtctgaatt ggtcagacaa tgtcttgtgt gcctggcata
4800

tgataggcat ttaatagttt taaagaatta atgtatttag atgaattgca taccaaatct
4860

gctgtctttt ctttatggct tcattaactt aatttgagag aaattaatta ttctgcaact
4920

tagggacaag tcatgtcttt gaatattctg tagtttgagg agaatatttg ttatatttgc
4980

aaaataaaat aagtttgcaa gttttttttt tctgccccaa agagctctgt gtccttgaac
5040

ataaaataca aataaccgct atgctgttaa ttattggcaa atgtcccatt ttcaacctaa
5100

ggaaatacca taaagtaaca gatataccaa caaaaggtta ctagttaaca ggcattgcct
5160

gaaaagagta taaaagaatt tcagcatgat tttccatatt gtgcttccac cactgccaat
5220

aaca
5224